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AMILTON B. G. ROBINSON

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EDITOR'S VIEWPOINT

English (p. 4) examined the salivary glands of rats after direct x-ray irradiation. Interest in this subject has been increasing and studies are being reported from different laboratories. English's observations suggest that the submaxillary glands are the best glands for study of x-ray irradiation changes in rats.

Sausen (p. 12) used radioactive calcium in a study of electrophoresis in dentin. He found that small currents would conduct the calcium ions through dog's freshly cut dentin within 60 seconds but that intact enamel was practically impermeable. Too much current resulted in marked pulpal damage. The calcium is gradually removed from the dentin. Radioactive materials are finding new applications in testing therapeutic agents, as in this study.

Bélanger (p. 20) observed the incorporation of radioactive sulfate into the growing enamel of rats and hamsters. Radiographs and sections of jaws stained by histochemical methods were compared. The sulfur appeared to be synthesized into acid sulfomucoprotein before keratinization and to disappear later. The substance, in quantity, seems to inhibit mineralization. It is proposed that sulfopolysaccharides, appearing at the dentinoenamel junction and diffusing outward at the maturation period, may favor mineralization.

Wainwright and Belgorod (p. 28) repeated the experiment of sealing substances in dentin and observing the penetration. However, they used radioactive-tagged substances and found that, in extracted teeth, the substance tested penetrated through the dentin to the dentinoenamel junction in about 20 minutes. In vivo studies of nontoxic "tagged" substances may give additional information on the exchange of ions in teeth, but care must be taken lest exchange be broadly interpreted as metabolism and, prematurely, as evidences of protective response or repair.

Myers (p. 38) used the polarizing microscope to study "chalky spots" in enamel. Since these are the regions which take up fluorides it was decided that their ability to imbibe fluids should be determined. Positive birefringence in islands within "chalky spots" indicate their ability for imbibition of fluids (presumably including fluoride solutions) and suggest that submicroscopic spaces present in the chalky regions, and not present in well-calcified enamel, permit imbibition in the former regions.

Lyon, Dickson, and Schoonover (p. 44) discuss the mechanism of hygroscopic expansion of dental casting investments. They propose that the so-called hydroscopic expansion is a continuation of normal setting expansion produced by introduction of additional water.

Phillips, Schnell, Johnson, and Phillips (p. 51) repeated previous work on electric conductivity of dental cement, using moistened specimens. They found, contrary to results with dry cements, that moist dental cements are questionable insulators against electric conductivity.

McCann and Bullock (p. 59), after studies of the reaction of fluoride ion with hydroxyapatite (*J. Biol. Chem.* 201: 247, 1953), proceeded to examine its reaction with powdered enamel and dentin. The reaction of dentin, and to a lesser degree enamel, proved to be much more complex from the reaction with hydroxyapatite. It appears that formation of fluorapatite is a direct reaction from fluoridated water but that topical application first results in formation of calcium fluoride which later may dissolve out, leaving fluorapatite and adsorbed fluoride.

Muhler and Day (p. 68) gave sodium fluoride, stannous fluoride, and stannous chlorofluoride to rats in their drinking water at different pH levels. Although at pH 3.5 the tin compounds were much more effective than the sodium salt, at pH 5.5, the difference was not as great, pH not influencing the sodium fluoride as greatly. A most interesting observation is the lack of correlation between fluoride storage (in bone) and protection against caries. Shafer and Muhler made a similar observation in their study of sex hormones and caries in the rat (*J. D. Res.* 33: 842, 1954).

Carter, Jay, Shklair, and Daniel (p. 73) did not find any significant reduction in dental cavity formation after application of 2 per cent sodium fluoride solution to the teeth of young women (Waves). Since there is still some question of the efficacy of this technic for adults, it would be well to use a group of children with the adults as an added control. Although it appears to be unlikely, one may raise the question whether these authors would have obtained significant reduction in caries activity of children, although others have consistently. It seems unlikely that topically applied fluorides will reduce caries by 40 per cent in youngsters and not at all in young adults.

Continued study of the children in Evanston who have been drinking fluoridated water for approximately 5 years showed about 4 per cent reduction in the caries rate per year. If this rate of reduction continues for 5 more years, the caries rate in Evanston will be approximately equal to naturally fluoridated Aurora's rate. Hill, Blayney, and Wolf (p. 77) present several approaches to analysis of their data.

Kitchin and Permar (p. 89) report their results after 8 years of conducting a caries control program through reduction in carbohydrate intake. The patients, for the most part, were advised by private dentists, and the lactobacillus count was used as a criterion of success. A total of 77 per cent of 531 patients successfully completed a 2-week carbohydrate restriction, 85 per cent of 376 patients showed low lactobacillus counts after a second 2-week program, and 74 per cent of 306 successfully completed a 6-week program. After 6 months to 3 years, 61 to 74 per cent still demonstrated low counts. Failures are accounted for by deviations from the program. This report indicates that, in the hands of private practitioners who are not specially trained, dietary reduction in carbohydrate intake is about 75 per cent successful as measured by the salivary lactobacillus index and, presumably, reduces caries activity to the same extent.

Laufer (p. 94) found the DMF experience in 13- to 17-year-olds in Tel Aviv to be one-half that of Philadelphia children and one-third that of New Zealand children. It is suggested that the difference in sugar consumption (30 pounds per capita per year in Tel Aviv vs. 120 pounds in the United States) may account for most of this difference. In spite of poorer oral hygiene, but with higher detergency of foods in Tel Aviv, gingivitis (by P. M. A.) was five times higher in Philadelphia than in Tel Aviv. While 13.3 per cent of Philadelphia carious teeth are lost by extraction, only 4.1 per cent of New Zealand teeth and 2.9 per cent of Tel Aviv teeth are extracted. In Philadelphia only 42 per cent of carious teeth are filled, 69 per cent in Tel Aviv, and 86 per cent in New Zealand. The latter are the results of school or public health programs. It would be most interesting to study the per cent of teeth left at 30, 40, 50, and 60 years of age. In other words, is the number of fillings placed in the teeth of children a good index of the number of teeth that will be preserved, or must we consider quality as well as quantity?

The statement appears in the literature that no area of the skin should be exposed to more than 600 M.A.S. in any 2-week period. Investigations of Richards (p. 100) lead him to suggest that 400 M.A.S. in any 2-week period is a more realistic figure, for 74 per cent of nineteen subjects developed erythema on the skin or the forearm at 600 M.A.S.

The determination of a chemical constitution of a tissue or fluid often appears as a simple routine testing procedure. This attitude, on the part of investigators who are not chemists, probably has led to many unwarranted conclusions. The saliva presents a difficult problem when its mineral content is to be determined. Ericsson (p. 104), while working as a Public Health Service Research Fellow, studied methods for determination of calcium and magnesium in saliva. He presents his findings and recommended technics.

Rosen, Benarde, Hunt, and Hoppert (p. 113) have extended studies of the Michigan State colonies of caries-susceptible and caries-resistant rats to their oral flora. Lactobacilli and *S. salivarius* occurred more frequently in rats of the caries-susceptible strains while an as-yet-unidentified streptococcus was found in all caries-resistant and 18 per cent of caries-susceptible rats. Heredity appears to influence the oral flora. It is highly important that these investigations be continued with full knowledge of past studies of oral flora of human and animal subjects.

Lucas and Thonard (p. 118) found cultures of organisms from the gingival pockets of 12 individuals with periodontitis and from the sulci of 6 with healthy gingivae capable of distintegrating collagen in vitro. The possible role of many different organisms in the production of periodontal disease is proposed.

Bevelander and Johnson (p. 123) using histochemical technics found that mucopolysaccharides increase in areas of calcification of enamel and dentin. The polysaccharide complex in the enamel organ appears to differ from that in the dentin-forming region. Until the mechanism of calcification is reevaluated, the authors hesitate to assign the role in calcification of polysaccharides.

Sognnaes and Lustig (p. 132) report histochemical findings on the "horny teeth" of lamprey. The oral structures of lamprey, representing one of the earliest evolutionary forms of toothlike structures, have been examined grossly and microscopically, previously. Histochemistry adds the information that their cornification is first associated with the appearance of sulfhydryl-containing granules and that the epithelial cells form a mucous product. The epithelial and mesenchymal cells do not specialize, as do odontogenic cells in higher forms. It is suggested that substances not present in the oral tissues of the lamprey are necessary for deposition of calcium phosphate.

Forscher, Paulsen, and Hess (p. 144) had demonstrated that the glycogen content of shallow periodontal pockets was higher than in healthy gingival tissues, but that as pocket depth increased the glycogen level decreased (*J. D. Res.* 33: 445, 1954). In order to overcome the possible objection that edema had altered the relative weights of the normal and inflamed tissues, they analyzed for tyrosine and glycogen and confirmed their earlier conclusion.

H. B. G. R.

MORPHOLOGIC EFFECTS OF IRRADIATION ON THE SALIVARY GLANDS OF RATS

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INTRODUCTION

THE marked physiologic response of salivary glands to radiation has received considerable attention; however, the less prominent histologic changes corresponding to those of function have not been adequately studied.[1, 6] This study pertains to microscopic anatomic changes which were observed in the salivary glands of white rats selected from three separate radiation experiments. One experiment was designed to determine acute radiation changes as a function of time, while the other two studies were performed on rats which were sacrificed 100 days following irradiation. The study is limited to the 1,000-1,500 r dose level (local). Other reports have or will be made on these same animals in relation to changes occurring in their dentition.[3] Normal rat salivary glands are described in some detail because this material is not readily available in the literature.

MATERIALS AND METHOD

The white rats used in each experiment were from the same strain of Osborne-Mendel breeder animals, but different generations were involved. The first and second experiments used littermate unirradiated animals as controls; the third experiment was a pilot study in which no control animals were provided. All animals were maintained on screen-bottomed cages, under good laboratory conditions from birth until termination of each study. The diets in the respective experiments were Purina Lab Chow, corn caries diet,* and Rockland rat diet, pellet form. Food and distilled water were available ad libitum, and the diets are not believed to be a factor in the study herein reported. Both sexes were used.

The irradiation procedures were similar in each experiment, except that in the second study 1,000 r was applied, whereas in the other two the dose was 1,500 r. A Picker commercial x-ray machine[1] was used, the physical factors being 200 KVP, 25 Ma., filters ¼ mm. Cu and 1 mm. Al, half-value layer 0.82 mm. Cu, target-skin distance 50 cm., cone 20 by 20 cm., roentgens per minute 44. The rats were exposed in groups of ten in special plastic boxes designed to retain animals in a fixed position. Only the heads and necks were exposed, the bodies being protected by lead sheeting.

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*Corn caries diet: 20 pounds Quaker yellow hominy grits, 10 pounds Klim, 1 pound alfalfa, ½ pound salt.

In the first experiment fifty rats were irradiated and fifty littermates were retained as controls. There was an equal division between the sexes in each group. The design of the experiment was to provide tissue for study at increasing intervals following exposure to x-rays. Due to the high mortality rate during the 7- to 9-day interval, the material was sparse beyond the 9-day period. The intervals were as follows: 16 hours, 24 hours, 39 hours, and in daily intervals at 3, 5, 7, 8, 9, 11, 16, and 21 days.

In the second experiment forty-four rats equally divided between the sexes were used, one-half of them being retained as littermate controls. This experiment was designed by M. G. Wheateroft for the study of radiation effects in relation to oral flora and dental caries incidence in rats.

The third study, involving eighteen rats, used a segment of material from an experiment designed by C. A. Schlack for the purpose of determining the lethality of head irradiation to rats. A previous report makes use of these and other rats as the basis of study.[2]

Animals were sacrificed by an overdose of chloroform anesthesia, and the salivary glands were promptly removed and placed in fixative. In the first experiment, fixation was in 10 per cent neutral formalin and Zenker's fluid. Material was embedded in paraffin, cut at 5 microns, and stained by the following methods: Harris' hematoxylin and eosin, Schiff's stain for mucin, Mallory's analin blue collagen stain, and Masson's trichrome connective-tissue stain. In the second and third experiments fixation was in 10 per cent neutral formalin, tissue was blocked in paraffin, cut at 5 to 7 microns, and stained in hematoxylin and eosin.

RESULTS

The preliminary study of the salivary glands from the three experiments consisted of an attempt to separate the irradiated salivary glands from those of the controls on the basis of morphologic changes observed. It was not possible to identify irradiated tissue from animals sacrificed during the first two weeks; however, consistent identification was possible beyond this period, that is, at 16, 21, and 100 days.

The specific salivary gland which consistently exhibited histologic changes following radiation was the submaxillary gland. This is a paired gland lying near the median line, just above the hyoid bone. It is the largest single mass of salivary tissue, and together with the greater sublingual gland forms a capsulated almond-shaped gland (Fig. 1, *a*). The submaxillary gland contains three easily identifiable structures: acini, tubules, and collecting ducts. The acini are smaller than the tubules, and consist of irregular masses of cells which are roughly pyramidal in form. The acinar cells contain round or ovoid nuclei measuring about 10 microns in diameter, located basally. This portion of the gland resembles the parotid gland acini in size and structure, and like the latter gland, has no central cavity or secretory space within the acinar mass. The nuclei are darkly basophilic, with quite evenly distributed chromatin, and usually contain one nucleolus. The cytoplasm in fixed preparations is some-

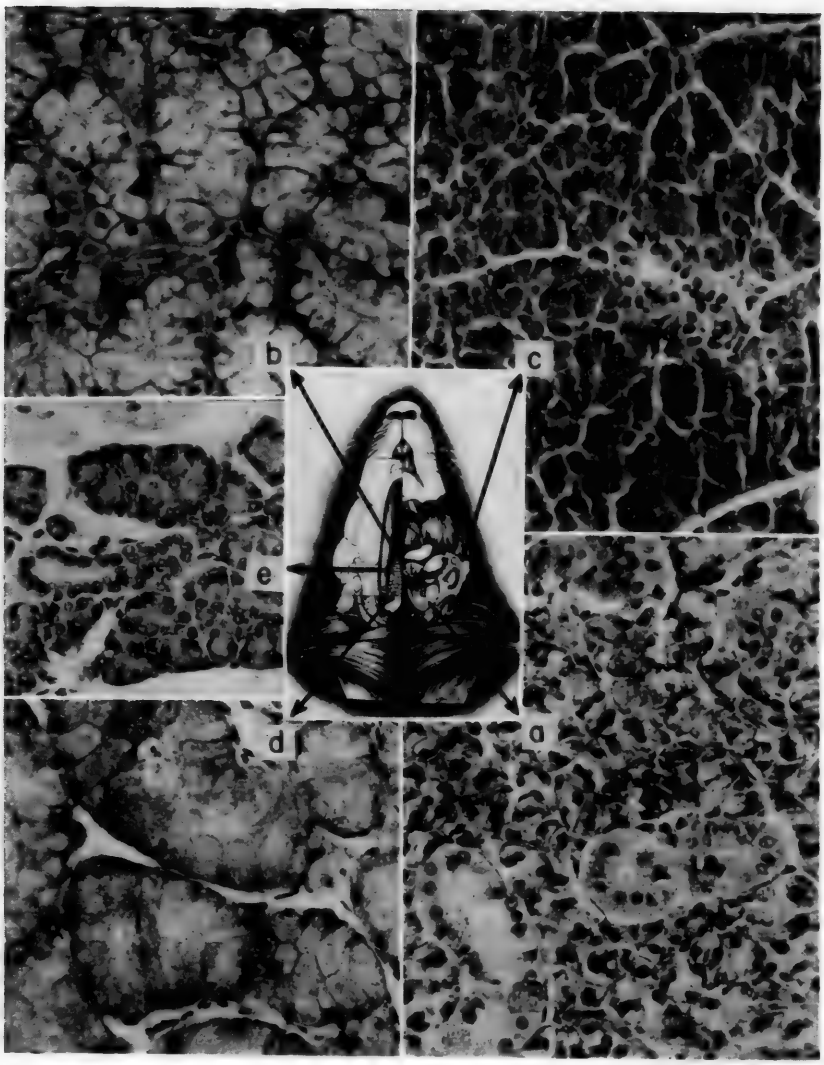

Fig. 1.—Salivary glands from normal rats. *a* and *b*: ×240; *c* and *e*: ×120; *d*: ×430. Reduced ⅕. (*a*) Submaxillary gland, (*b*) greater sublingual gland, (*c*) parotid gland, (*d*) mucous gland of tongue, (*e*) serous gland of tongue, (*f*) lacrimal gland. Other unlettered oval structures in central drawing are lymph nodes. Structures *a* and *b* are often within same capsule and cannot be grossly identified as separate structures. Parotid gland (*c*) is not discrete mass, but is distributed from lacrimal gland (near ear) to submaxillary gland which is near the mid-line. Note that salivary glands of tongue are similar although not identical to neck glands. (Drawing by Otho Hon.)

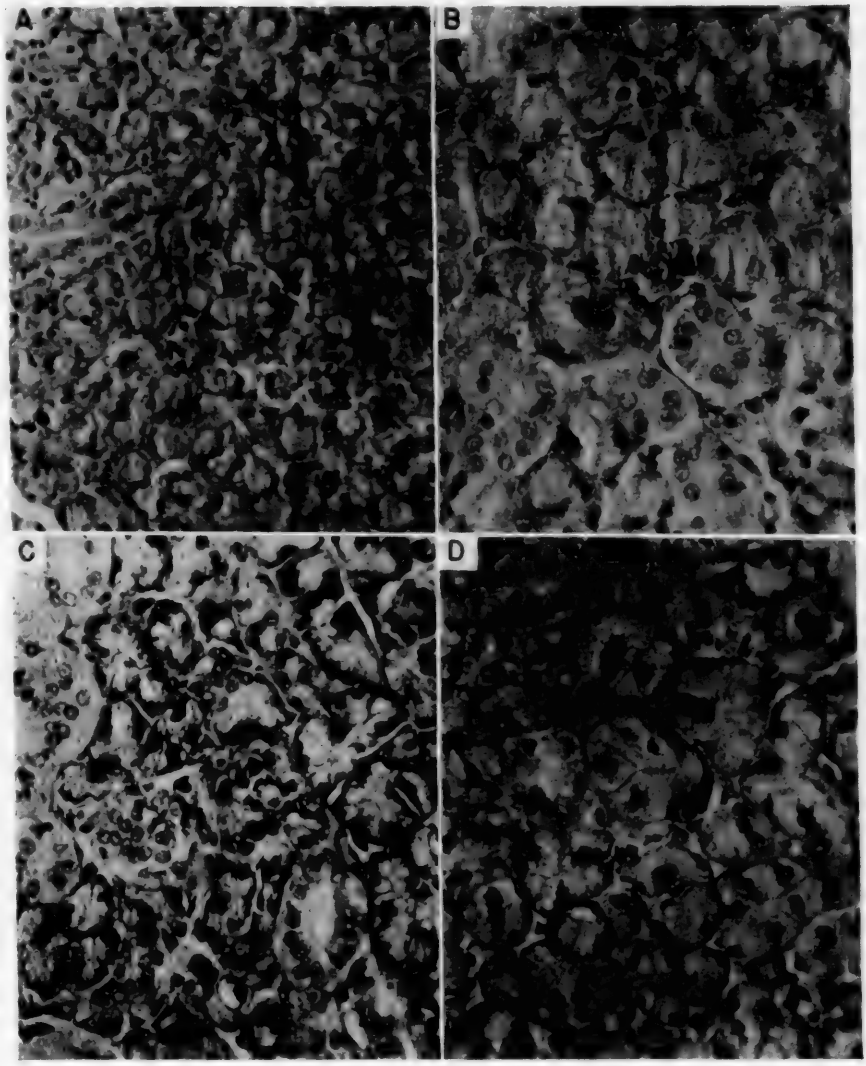

Fig. 2.—Submaxillary salivary glands from irradiated rats. *A:* ×120; *B*, *C*, and *D:* ×240. Reduced ⅓. *A* and *B* from first experiment, *C* from second experiment, and *D* from third experiment. Note that in relatively low magnification (*A*) enlargement of nuclei is prominent characteristic. In higher magnification shown in other sections, nuclei are often large and irregular in shape. Most prominent characteristic at high magnification is the peculiar patterns formed within acini due to the random alteration of a few cells per acini. One or two cells will be greatly enlarged and have odd-shaped, darkly staining nuclei, whereas other cells will be either of normal size or small. Nuclei may be either larger or smaller than normal, and some acini appear to have less than the usual number of nuclei. Less specific changes may also be seen.

what irregularly distributed, faintly basophilic with an acidophilic background. With Schiff's stain, the cytoplasm of the acini has a granular appearance and is a dull rusty-purple in color (in strong contrast with the bright red-purple of the mucin-containing greater sublingual gland, which lies adjacent). The tubules consist of cuboidal or low columnar cells, with quite centrally placed nuclei of good size (about 15 μ). The structural details show very well by Schiff's stain, for the chromatin granules of the nuclei and the nucleolus stain black, the cytoplasm granules stain yellow, and the large secretory granules produced by the tubules take an orange color. A definite central open space exists within the tubule. The collecting ducts are similar to the tubules in staining characteristics, the main differences being that the ducts are larger, have a larger central open space, the cells are tall columnar with vertical striations basally, and no mucinogen granules are produced.

The radiation changes observed in the submaxillary gland were sufficient to be noted at relatively low magnification (×100) and consisted of variability in size and staining characteristics of the nuclei of the acinar cells (Fig. 2). A detailed examination at ×200 and ×475 magnification revealed extensive nuclear pleomorphism and a variation in nuclear size of from 5 to 30 microns. Many of the nuclei which were abnormal in size also took a heavy basic stain in hematoxylin and eosin, which emphasized size variability. The peculiarly shaped nuclei included stellate and discoid forms, which frequently stained heavily with hematoxylin and were thus very noticeable. However, the three nuclear changes described did not always occur simultaneously. Size of whole cells was not easily discernible, for the cellular membranes were indistinct. Frequently, however, when the nucleus of an alveolar cell was large, there was an equal increase in cytoplasm about the nucleus, and these "giant" cells were optically prominent. Changes in the tubules and ducts of this gland were not found to be consistent enough to be reliable indicators of radiation damage. Often one had the impression that architectural disarrangement or actual epithelial dislodgement of cells was a characteristic of the exposed gland, but the same condition was sometimes found in control animals.

Other glands of the salivary group which were studied include: the greater sublingual gland (Fig. 1, *b*), the parotid gland (Fig. 1, *c*), the mucous and serous glands of the tongue (Fig. 1, *d* and *e*), and, in an incidental fashion, one nonsalivary gland, the lacrimal gland (Fig. 1, *f*). The minor sublingual glands were not studied. Inasmuch as no consistent changes of a reliable nature were found in these glands, only brief comments will be made.

As has been indicated, the greater sublingual glands lie in depressions at the superior-lateral-anterior poles of each of the submaxillary glands (Fig. 1, *b*). They are composed of acini that are several times larger than those of the submaxillary gland and are of a mucous-gland variety. The cells of the acini appear to be of two types. Most large acini contain twelve to twenty cells in section, with nuclei measuring about 10 microns which are basally disposed and deeply basophilic in staining characteristic. The cell membranes form a network to-

ward the center of the acini, revealing the pyramidal form of the cells. Unless stained with a mucin stain there is considerable clear space in each cell. A second type cell may be found that has a nucleus two to three times larger than the first, and is encompassed by a zone of quite heavily staining cytoplasm. These cell types frequently occur in the large acini, but more often intermingle with small acini or occur singly or in small groups alone. With special stains, as Schiff's or mucicarmine, their cytoplasm absorbs the glycoprotein specific stain as well as does the cytoplasm of the other acinar cells. In the irradiated rats it was at first believed that these larger cells occurred more frequently and were more varied in size than in control animals. When an attempt was made to use this gland alone in determining whether or not animals were irradiated, it was found to be an unreliable means of identification.

The mucous glands located in the distal portion of the tongue of the rat are quite similar to the greater sublingual glands in general structure (Fig. 1, *d*). In addition to the fact that muscular strands can be found in most ×100 fields, these glands can be differentiated from the latter by their finer cytoplasmic structure within mucous acini, the relative infrequency of the larger variety of cells which has been described, and by the presence of some very large mucous acini in most fields. The irradiated mucous glands of the tongue exhibited no characteristics that were sufficiently constant to serve as a means of separating irradiated from control material.

The parotid glands of the rat and the serous type glands of the mid-portion of the rat tongue are nearly identical in basic structure (Fig. 1, *c* and *d*). The acini are compact masses of about eight to fourteen pyramidal cells in section, which contain basally located rounded nuclei. The chromatin of the nuclei tends to be concentrated into numerous darkly staining masses, and the rest of the cell is densely packed with secretory granules that are primarily eosinophylic in hematoxylin and eosin, and rust colored in Schiff's stain. The ducts in well-fixed preparations are composed of columnar epithelial cells with basally located round nuclei that are slightly larger than acinar nuclei (about 15 μ). Unless the glands of control animals are cut in very small pieces, or a rapidly penetrating fixative is used, the ductal epithelium is considerably altered in the parotid gland: the cytoplasm is sloughed off and the nuclei are shrunken to one-half the normal size. Similar changes are common following irradiation, as well as vacuolation of acini, pyknosis, fragmentation of cells, and disorganization of the architectural pattern. Since all these changes can result from various (often unknown) causes, we were not consistently able to separate irradiated from control animals on the basis of such changes in the parotid glands even though it is felt that these changes can be legitimately included under radiation manifestations.

The only gland exhibiting changes which could consistently be used as a morphologic means of positively separating experimental from control material was the submaxillary gland. Here the acinar nuclei were varied in size from −100 per cent to +300 per cent, nuclear staining was altered, and nuclear pleomorphism was prominent.

DISCUSSION

A brief review of the literature pertaining to radiation changes observed in salivary glands revealed that not many careful or extensive studies have been made. The changes described in many instances were of a kind that could be elicited by many causes, which gave reason for considerable caution in placing too much significance on them. For example, there is a reference to "degeneration of some epithelial cells lining acini . . . reduction of amount of mucin stored in alveolar cells . . . lymphocytic infiltration around secretory ducts . . . proliferation of connective tissue in the stroma,"[1] all of which were changes that could be found to some extent in salivary glands of animals that had no experimental treatment other than sacrifice and preparation of tissues for microscopic examination. In order for these changes to be significant, some quantitative evaluations must be established, which presents no easy problem in morphologic studies. Other changes described in a review article[6] were physiologic or of a nature that was extremely hard to evaluate, such as "stromal edema . . . reduction of salivary flow . . . fatty degeneration . . . hyperemia of blood vessels." Separation of unmarked tissues on the basis of changes observed should perhaps be the first test applied. An evaluation of changes which occur before morphologic alteration is observed, as well as a better correlation between physiologic and morphologic effects of radiation, may be provided by combining histochemical and enzymatic tests to the described procedures.

The work of Lazarus and Barlow referred to by Friedman[6] indicated that serous glands are more resistant to radiation damage than mucous glands, as indicated by morphologic change. Our present paper as well as previous work[5] is somewhat in agreement with this, but the mixed gland (submaxillary) is the least resistant. Recently it has been reported by Shafer[7] that parotid glands show the most pronounced changes; however, the dose eliciting the changes was considerably higher than ours, and the posttreatment period was twenty-one days.

An interpretation of morphologic changes such as are described in this paper as occurring in submaxillary glands of rats is quite hazardous even though the consistency of these changes is extremely regular. The fact that the nuclear alteration provides the prominent means of diagnostic identification of exposed glands should not lead one into the assumption that such change is necessarily the most important. Study of the mitochondria and smaller particles of the cytoplasm by the methods described is relatively inadequate; therefore, equal or more important changes may occur here that do not lend themselves to visual estimation. We are hoping that our current use of biochemical methods in the study of separated cell components of salivary glands will fill in details not available by morphologic techniques.

The time factor of sixteen or more days for the enlargement or diminishing of cells in size is long enough that growth mechanisms may be involved in the basic process. Hyperplasia or hypoplasia are well-worn sequences in injury of tissues. One might also picture these changes in cellular size as part

of a process of degeneration, leading to random necrosis or fibrosis. Other studies on dogs[7] show without doubt that salivary glands do become extremely fibrosed, atrophic, and, after periods as long as six months, develop characteristics frequently associated with malignant tumors (for example, cells become polymorphic, show decreased differentiation, show changes in staining characteristics and architectural arrangement). Cells that survive, even when rather bizarre in form, often appear to be able to produce secretory material that is normal to the extent ascertainable by using special stains for mucin. Whether there is an actual alteration in the chemical composition of the secretory products of these glands, or a change in their enzyme systems as a result of radiation, may be revealed by further histochemical and biochemical investigation.

SUMMARY

Salivary glands of rats were examined sixteen hours to 100 days following application of 200 kv. x-ray radiation in doses of 1,000 and 1,500 r. A total of 162 rats were involved, seventy-two of them being used as littermate controls. Sufficient morphologic changes were observed in submaxillary salivary glands of animals sacrificed beyond the fifteen-day period to permit accurate separation of unmarked control and irradiated tissues. The changes consisted primarily of alteration in size, shape, and staining characteristics of nuclei of acinar cells. Changes were observed in other salivary glands, but were not characteristic enough to serve as invariable means of identification.

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ELECTROPHORESIS IN DENTIN WITH RADIOACTIVE CALCIUM

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ELECTROPHORESIS has a long history of usage in dental treatments, continuing for nearly one century.[1] Today, its use is almost exclusively restricted to root canal therapy. Its efficacy in this regard is not established with complete precision, nor is there widespread accord on the merit of its routine use in dental practice for any purpose.

W. J. Morton[2] proposed sterilization of dentin with zinc ions as early as 1896. The value of this treatment was strongly supported by several investigators in Europe[3-5] and later, by Grossman and Appleton,[6] who conducted extensive studies on the antibacterial activities of various ions with electrophoresis. A wide variety of ionic agents have been proposed for different oral applications[7]; however, the actual path and depth of movement for these agents had not been demonstrated. The calculations by Price[8] and Adler[9] seemed to indicate that a deep penetration could not be accomplished very quickly, and the literature[10, 11] has reflected a growing opinion that electrophoresis is an ineffective and tedious procedure. Such opinion gradually eliminated this process from the treatments of sterilizing and bleaching dental tissues, but a recent demonstration[12] that the saturation of dentinal tubules with calcium fluoride may be useful has revived this question. Accordingly, this matter has been studied, using radioactive calcium (Ca^{45}) as a key instrument, because of the decisive certainty of its results and the vivid way in which the autoradiograph illustrates the process in a direct visual recording.

This work is principally concerned with the determination of the effectiveness of electrophoresis in developing a high concentration of exogenous ions in dentinal tubules. The scope of this investigation necessarily had to include an analysis of basic factors inherent in the process of electrically propelled movements, such as: concentration of the electrolyte, the amount of current applied, and the duration of the treatment. Also considered are the physical dimensions and structural arrangement of the involved tooth structure, as well as its composition and the response of vital tissue to the treatment.

EXPERIMENTAL PROCEDURE

All operations were performed on the teeth of four healthy, young, female dogs. These animals were administered pentobarbital sodium intraperitoneally and then placed on an electrically insulated table. Posterior teeth were chosen for their greater bulk of dentin and their greater distance from corresponding control teeth of the opposite side. The large canines were used only when pulp tissue studies were planned.

The Ca^{45} used in this study was obtained from the Oak Ridge National Laboratory on allocation from the Isotope Division of the U. S. Atomic Energy Commission.

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Design of the electric circuit was a simple series arrangement including: a battery of dry cells as a source of direct current, a coil type ammeter with a sensitivity of 2 microamperes, a bank of rheostats, and the two electrodes. A stainless steel disc 2 inches in diameter constituted the cathode. This was always placed directly against cheek mucosa, opposite to the side of operation. Pure platinum wire was used for the anode. This was fitted with a plastic adapter to one end of a small glass tube and projected through nearly the full length of the glass. The other end of this tube was drawn out to capillary size, narrow enough to fit within a cavity of a small tooth (Fig. 1). The platinum wire could never touch tooth structure.

The anode tube was filled to the correct volume by aspiration, then the electrode was placed in it. Volumetric levels were measured in tenths of a cubic centimeter, providing for equal volumes of fresh solution to be used in each treatment. These solutions all possessed an activity of 0.01 mc. per milliliter, and the salt concentrations of calcium chloride were: 0.05 (carrier free), 0.1, and 1.0 normal.

Fig. 1.—Photograph of operative anode tube.

Shallow circular cavities were prepared in the buccal surfaces of selected teeth with a No. 36 inverted cone bur. These were cut to a consistent width and depth, with the intention being to remove a minimum of dentin. The capillary end of the filled anode tube was then placed against the dentin surface. As this break in surface tension occurred, the solution would fill the cavity. Cavity margins could not approximate gingival tissue too closely or the current would be short-circuited in this direction upon contact.

A flow of current was indicated by the milliammeter the instant the cavity was moistened. At this time, the circuit was kept closed for only one-second intervals while rheostat adjustments established the desired current level. Then the circuit was closed for the measured time of treatment, accurate to one second. Thus, three principal components of electrophoresis were controlled and, as each was varied separately, the constant values assumed for the others were: solution concentration — 0.1 normal, current strength — 0.5 milliampere, and duration of treatment — sixty seconds. After a treatment was completed, the cavities were blotted dry immediately and filled with silver amalgam. Postoperative time studies were so arranged that all extractions were made on the day of sacrifice.

The extracted teeth, used for pulp tissue study, were fixed with formalin, decalcified with 5 per cent nitric acid, embedded in celloidin, cut to a thickness of 12 μ, and stained with hematoxylin and eosin.

The remaining teeth were embedded in a maxtrix of clear methyl methacrylate plastic resin, preparatory to being sectioned. They were cut in a plane which bisected both cavity and pulp chamber as closely as possible. This surface was polished, and then the uncut side was held against a rotating disc of fine flint paper until the uniform thickness of 500 $\pm$ 10 μ was obtained. This was sufficiently thin to be placed in a photographic cassette wherein the section contacted a dental occlusal plane film. Exposure periods from eighteen to seventy-two hours were necessary to produce clear images of radioactive calcium distribution, with a reasonably higher density than background fog. To aid interpretation through orientation, a double exposure of the film was made with soft x-radiation, superimposing an image of the tooth outline.

The resultant autoradiographs were used for all qualitative observations on the treatment results. Any quantitative comparisons had to be judged by the density of images at corresponding areas or spots on the autoradiographs. This purpose was served with a special light meter in which a photoelectric cell recorded the amount of transmitted light being focused through the autoradiograph. All specimens thus compared were prepared on one plate of film; the middle portion of the density scale was used; and all readings were reproducible by this method.

RESULTS

Path of Penetration.—Several preliminary trials revealed that a treatment of 0.5 milliampere of current for one minute with a calcium concentration of 0.1 normal would move calcium ions through dentinal tubules to the pulpal wall. (Hereafter, this will be referred to as the "standard treatment.") The intensity of activity always was seen easily and was distributed uniformly. Only those tubules involved in the cavity preparation conducted exogenous calcium. The limits of this boundary were never violated, with rare exceptions being made in a slight penetration along the dentoenamel junction.

Simple Diffusion.—These limits also applied in cases where simple diffusion was employed and no electric potential was imposed. Simple diffusion required between seven and eight minutes to reach the pulpal wall (a distance of only 1.5 mm.) if the same hypotonic concentration (0.1 N Ca) used in the standard treatment was applied. However, this time was reduced to five minutes if the hypertonic concentration of calcium (1.0 N) was used.

Concentration of Solutions.—When electrophoresis was employed, the increased concentration of solution was of even greater value. With 1.0 normal solutions, a complete penetration to the pulpal wall was accomplished in only thirty seconds (Fig. 2), or only one-half the time required in the standard treatment. Furthermore, the intensity of activity in the tubules was increased by a factor of 2.5 over results with the lesser concentration. This may seem to conflict with the fact that greater electric conductivity occurs in solutions of lesser concentration, but this evidence rather illustrates that simple diffusion is

an active ally with electrophoresis, and a high concentration gradient is more useful (for short paths of movement) than the better electric conductivity obtained with more dilute solutions.

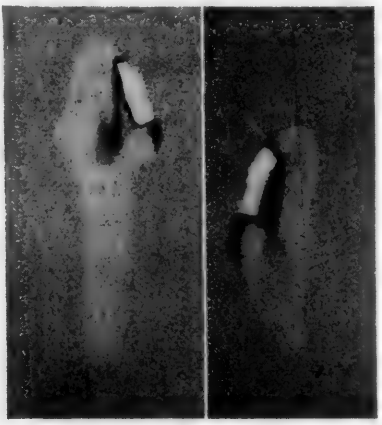
Accumulation of Ions.—Electrophoresis increased the concentration of exogenous ions within their path of movement. This cannot be brought about with simple diffusion because the intensity of activity in the dentinal path does not increase after the pulpal wall has been reached. When a current strength of 0.5 milliampere was applied, a concentration of radioactive calcium was built up very quickly within the tubules, reaching over 80 per cent of the ultimate concentration in three minutes. Then the rate decreased, with a slow improvement continuing to the maximum measurable concentration in approximately forty minutes.

Fig. 2.—Autoradiograph showing penetration from treatment of 0.25 milliampere-minute with a hypertonic solution of calcium.

It was observed that this accumulation of ions always proceeded in a characteristic pattern. The initial penetration through the full length of the tubules took place in a uniform manner but, if the treatment was continued, an area of increased density was seen first at the pulpal wall of the pulp chamber. This accumulation progressed along the lateral borders of the path, slowly spreading throughout all the tubules, which gradually became heavily saturated with the exogenous ions. Fig. 3 illustrates two stages of this process. One specimen (*A*) shows the increased density at the pulpal wall and lateral borders of the path. The other (*B*) is completely saturated to a point which represents the maximum measurable concentration with this autoradiographic technic.

Milliampere-Minutes.—Any one stage of treatment can be duplicated by either a low current strength for a longer period of time or a high current for a shorter time. The product of current strength and time, usually expressed

in the unit of milliampere-minutes, can serve as a valid scale of the "extent" of an electrophoretic treatment. When dealing with pulp tissue, though, this expression becomes more a theoretical than a practical consideration, largely because of the limited range of current strength tolerated by the pulp.



A. *B.*

Fig. 3.—Autoradiographs showing partial (*A*) and complete (*B*) saturation of tubules with calcium.

Fig. 4.—Autoradiographs showing distribution of calcium along canal walls after long treatments.

Movement Into Pulp Tissue.—It was interesting to observe that several autoradiographs demonstrated activity all along the pulpal wall present in the section. Fig. 4 shows two examples of this effect, which appeared in varying degrees on every specimen which received a treatment of 1.5 milliampere-minutes or more. A heavier ring of activity is seen all around the pulp chamber and level with the supplying dentinal tubules, then decreasing in intensity as the apex is approached. It may be significant that no tubules throughout the teeth allowed entry of calcium ions from their pulpal ends. Any further ionic movement, then, must have taken the calcium through the apex.

Time Disappearance.—A number of teeth were not extracted until varying periods of time after treatment, with the intention of learning the degree to which the exogenous calcium was sealed into a cavity by an amalgam restoration and also to determine the effectiveness of postoperative diffusion in eliminating this calcium from the tubules pulpally. All were given the standard treatment which consistently delivers a clear path of calcium to the pulpal wall. As seen in Fig. 5, *A*, this path apparently is undisturbed after four days. Most of the loss occurs within the next ten days, after which the residual calcium remains fixed during the next two-week period. This study was not followed longer than four weeks. Fig. 5, *B* demonstrates that the principal loss of calcium has occurred from the pulpal side of the cavity base. In contrast, the peripheral outline of the cavity has lost no continuity. Activity of Ca^{45} is reduced about 13 per cent in one month, but this amount of radioactive decay does not account for the difference between the two specimens in Fig. 5.

A. *B.*

Fig. 5.—Autoradiographs showing postoperative disappearance of calcium with time. Intervals shown are four days (*A*) and four weeks (*B*).

Treatments on Sound Enamel.—Several applications were made on the enamel surfaces of uncut teeth, simulating the procedure of a routine electric vitality test. The enamel plate was only 0.5 mm. thick, yet only very small currents could be obtained with the maximum potential (30 volts). This testifies to the high electric resistance of enamel in general. The milliammeter also demonstrated a consistent, but sporadic, current flow, indicating that the current may have been discharging directly through the enamel wall rather than being carried by ionic transfer. With 0.6 milliampere for four minutes there was no

penetration through enamel except at two very fine lines extending to the dentoenamel junction. No activity could be seen within underlying dentinal tubules with this treatment or with treatments of longer duration.

Current Strength.—If 30 volts were applied to a dentin surface, the resultant current would be much higher. Current strength is by far the most limited factor under control of the operator. It has been shown that the standard treatment, with a current of 0.5 milliampere, can accomplish complete penetration of calcium to the pulpal wall, but a current of 0.4 milliampere will create only a partially complete path within the time limit, and less current strength will result in no observable movement from the cavity base in one minute.

Fig. 6.—Photomicrograph showing vacuolization in odontoblasts from high current strength.

Pulp Tissue Response.—At the other extreme of current strength the range is equally limiting. It was observed several times that when a high potential is applied the strong current level which is obtained gradually diminishes to a much lower value. This response was presumed to be a pathologic loss of conductivity in pulpal tissue. One specimen was intentionally subjected to a 30-volt potential which was applied until the initial current strength (1.25 milliamperes) was reduced by one-half. This required only two minutes, then the tooth was extracted immediately.

As seen in Fig. 6, the pulp contents were distorted into an axial orientation of fibers and bands of morphologically indistinct material. All odontoblasts appeared slightly shrunken, but those involved under the cavity preparation exhibited characteristic differences. Small intracellular vacuoles formed which enlarged and coalesced together to form the large vacuole seen in the photomicrograph. These blisters eventually burst apart to leave the area stripped of all odontoblasts. No disturbances of odontoblasts were found in

teeth which had received the standard treatment. This demonstrates the very critical importance of controlling current levels because of the great sensitivity of substrate tissues.

SUMMARY AND CONCLUSIONS

Electrophoresis treatments were performed on the teeth of four dogs. Different concentrations of radioactive calcium chloride solutions were placed in shallow cavities and submitted to a direct current for varying amounts of time and current strength. Several treatments were made on the enamel surfaces of uncut teeth, and other timed applications were made on both enamel and dentin without any current. When a treatment was completed, the cavity was filled with silver amalgam, and the tooth was extracted immediately or after a measured period of time.

Pulp reactions were studied by histologic sectioning of selected teeth. The remaining teeth were invested in a clear plastic and ground to a thickness of 0.5 mm. Autoradiographs of these sections were used to determine distribution of the calcium. Relative concentrations of activity were measured by a photoelectric cell on a beam of light passed through the autoradiographs.

The following conclusions are bound by the limits of experimental procedure and by their application to dogs' teeth:

1. In a dog's tooth, electrophoresis will move a 0.1 N concentration of calcium ions through the full length of the tubules within sixty seconds if a current strength of 0.5 milliampere is properly applied.

2. Electrophoresis will move a hypotonic concentration of ions against an unfavorable concentration gradient. A favorable gradient will assist electrophoresis at critical levels of treatment.

3. Electrophoresis will increase the concentration of supplied ions in areas of lower secondary conductance, such as in dentinal tubules.

4. Within the threshold of pulp tissue tolerance, electrophoresis will not move calcium ions through enamel, and this tissue is a poor conductor of current.

5. The electrophoretic saturation of tubules with exogenous calcium ions will remain for at least four days if the cavity is sealed with silver amalgam.

6. An intracellular vacuolization of odontoblasts will occur at the end of conducting tubules if a current strength greater than 1 milliampere is applied.

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AUTORADIOGRAPHIC DETECTION OF RADIOSULFATE INCORPORATION BY THE GROWING ENAMEL OF RATS AND HAMSTERS

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INTRODUCTION

THE formation of enamel seems to be the result of two different operations: growth and maturation. The period of growth is characterized by the formation of an insoluble organic matrix with incorporation of a proportion of mineral salts. During maturation, the organic substance decreases considerably, and the mineral constituents attain their adult concentration.[1] Matrix deposition has been attributed to the secretory activity of the ameloblasts, and a distinction has been made between the young, large ameloblasts, considered to be secretory, and the older, short ameloblasts, which are said to be absorptive.[2, 3] The organic matrix has been identified as keratin[4-7] on the basis of chemical analysis, tinctorial comparisons, and polarized light microscopy. Pincus[8, 9] has described it as a mucoprotein because of its low nitrogen content and its yielding of hexosamine. Wislocki and Sognnaes[10] have reported the presence of acid polysaccharides and sulfydryl groups, as well as other organic constituents detected by histochemical staining.

The present work has been carried out in order to trace the organic fixation of sulfate in the body and to study the relationship between organic sulfate deposition and previous autoradiographic mapping of mineralization.[11-14]

MATERIALS AND TECHNICS

Rats of 4, 5, 6, 7, 8, and 10 days of age were injected subcutaneously with a single dose of 5 μc per gram of S^{35}-separated isotope, obtained as weak H_2SO_4. These animals were sacrificed at intervals of one and two hours and one, two, four, six, and eight days thereafter. A series of six hamsters of 4 days of age were treated similarly and sacrificed at intervals of two hours, and one, two, and four days. After removal of most of the soft tissues, the heads were bisected and fixed in a mixture of ⅓ neutral formaldehyde and ⅔ 95 per cent ethanol. They were embedded in low viscosity nitrocellulose and cut 10 to 12 μ without demineralization. The heads of 12-day-old rats were embedded in polyester thermosetting resin (Ward's Bio-Plastic) and ground mechanically to a thickness of approximately 25 μ. Autoradiographs were obtained by the modified inversion technic.[15] For purposes of comparison, some sections were demineralized on the slide in a mixture of formic acid and sodium citrate at a pH of 4.9 for twenty-four hours, and others were sub-

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mitted to testicular hyaluronidase hydrolysis. This procedure was carried out in a 0.1 per cent solution of enzyme buffered at a pH of 5.6 at the temperature of 38° C. and for durations of one to three hours. After these various operations, the sections were washed in several changes of distilled water and prepared for autoradiography. On the other hand, preparations of this material were stained with toluidine blue, for metachromasia. A few hemisections of the heads were fixed in 80 per cent cold ethanol and treated by a slight modification of the Greep, Fischer, and Morse technic,[16] with rapid demineralization on the slide.[17]

Sections of mineralized and demineralized teeth were also stained by the ferric-ferricyanide technic of Chèvremont and Frédéric[18] for sulfydryl groups and counterstained for ten minutes in 0.02 per cent basic fuchsin, followed by rapid ethanol dehydration.

OBSERVATIONS

The rats of 4 days of age, sacrificed one or two hours after the introduction of the tracer, showed an intense linear reaction at the outer border of the enamel of the first molar. The reaction was more intense than that seen over the underlying dentin. The more primitive second molar had not yet grown any enamel and showed a reaction over dentin only. The first molar of the 4-day-old hamster is more mature than that of the rat of the same age. In these animals (Fig. 2) the autoradiograph of enamel growth did not extend to the apex of the tooth primordium but was limited to a relatively young area of enamel organ in which the ameloblasts were the highest.

In rats of 8 days of age sacrificed two hours after the injection of S^{35} (Fig. 1), a similar limitation of the extent of sulfur fixation was recorded. The area of sulfur fixation for each cusp was apparently inversely proportional to the age of the tissue. With longer exposures, a minor reaction was detected over the ameloblasts lining the zone of sulfur fixation (Figs. 1 and 5).

The outer limit of the area of sulfur fixation, on molars and also along the incisor (Fig. 6), was marked by a change in the ameloblasts: these became suddenly shorter, they stained more deeply, their processes were retracted, and frequently they became raised above the enamel surface for a short distance, after which they became closely attached again.

In the region of initial growth of enamel, particularly along the incisor (Figs. 7 and 8), a similar raised, dense area of ameloblasts could be seen. The cells of that area (Fig. 8) were also devoid of Tomes's processes, which appeared and became progressively longer with the beginning of enamel deposition. Likewise, S^{35} fixation (Fig. 7) was initiated along the modified, enlarged ameloblasts.

After twenty-four hours, the initial surface autoradiograph was still strong, but there was also some diffusion toward the inner regions of enamel. After two days (Fig. 3), the radiosulfur originally deposited at the surface of enamel had become displaced inward both by diffusion and by additional surface growth. A wide band of greater intensity was seen at a distance from

the surface. This distance was inversely proportional to the age of the enamel and represented, most probably, the portion of enamel laid down during the period of highest radioactivity of the blood.

At later intervals, the autoradiograph had appeared closer to the dentinoenamel junction, and at the same time it had become more diffuse and less intense than that seen over dentin, which in the original illustration was less intense than that of enamel. At six days, practically no picture was recorded over enamel, while the dentin, bone, and cartilage were still positive.

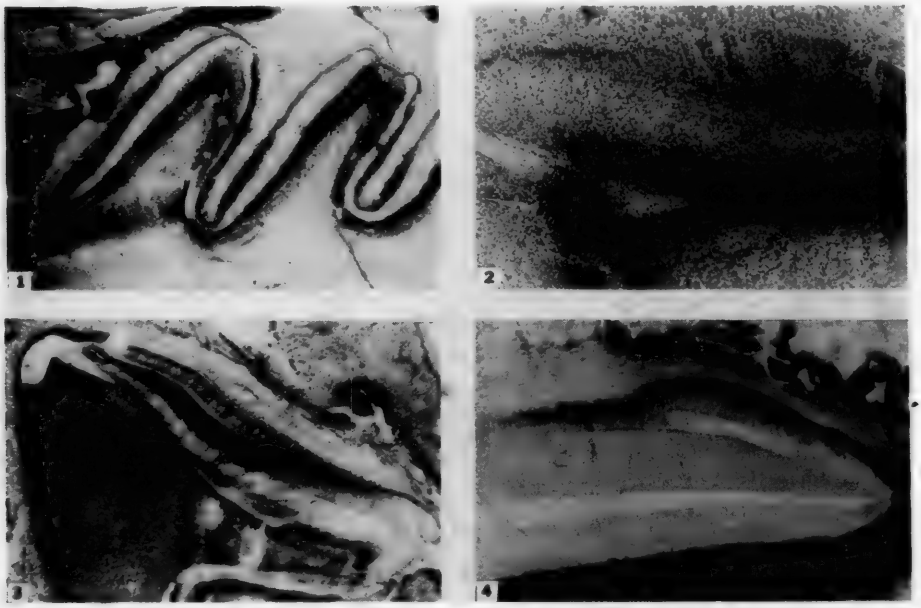

Fig. 1.—First molar, upper jaw, of an 8-day-old rat, sacrificed two hours after a subcutaneous injection of radiosulfate. Inverted autoradiograph (×40; reduced ⅓). The area of growth of the three cusps is of unequal length. It shows a deposit of S^{35} at the surface of enamel and over adjacent ameloblasts. There are also strong reactions over dentin and adjacent alveolar bone; also a mass of radioactive material in cervical region of pulp.

Fig. 2.—Part of the first molar, upper jaw, from a 4-day-old hamster treated as described previously. Inverted autoradiograph (×160; reduced ⅓). There are similar localizations in enamel and dentin. Picture in enamel is seen in relation to higher ameloblasts.

Fig. 3.—Part of first molar, upper jaw, from rat 7 days of age, sacrificed two days after subcutaneous injection of radiosulfate. Inverted autoradiograph (×70; reduced ⅓). The sulfate has diffused into substance of enamel proportionately to age of tissue. Linear area of heavy concentration is seen throughout area of intake. Dentin shows linear deposit at some distance from odontoblasts. Cervical region of pulp shows an accumulation of radiosulfate, in a pattern similar to that in Fig. 1.

Fig. 4.—Part of the first molar, upper jaw, in 12-day-old rat. Alkaline phosphatase reaction, showing that the enzyme is present over stratum intermedium next to ameloblasts in area of growth. Adjacent alveolar bone and dental pulp also shows a positive reaction. Greep-Fischer and Morse technic (×120; reduced ⅓).

The first molars of the 12-day-old animals were almost ready for eruption. In these teeth of animals sacrificed two hours after the S^{35} injection (Fig. 9), there was no reaction as described previously at the outer surface of the enamel. On the other hand, at the apex there was a fairly strong picture at the dentinoenamel junction and a diffuse picture progressing from this area

toward the surface. The more immature second molars in these animals have recorded an autoradiograph of surface intake like the first molars of younger animals.

Effect of Acid Demineralization.—Sections of tissues from animals sacrificed at the two-hour interval were submitted to acid demineralization on slide. The autoradiographs obtained from these (Fig. 5) showed the same picture over enamel, but the large over-all reaction over dentin had disappeared, and only a minor image has been recorded over predentin with some accumulation at the predentin-dentin border.

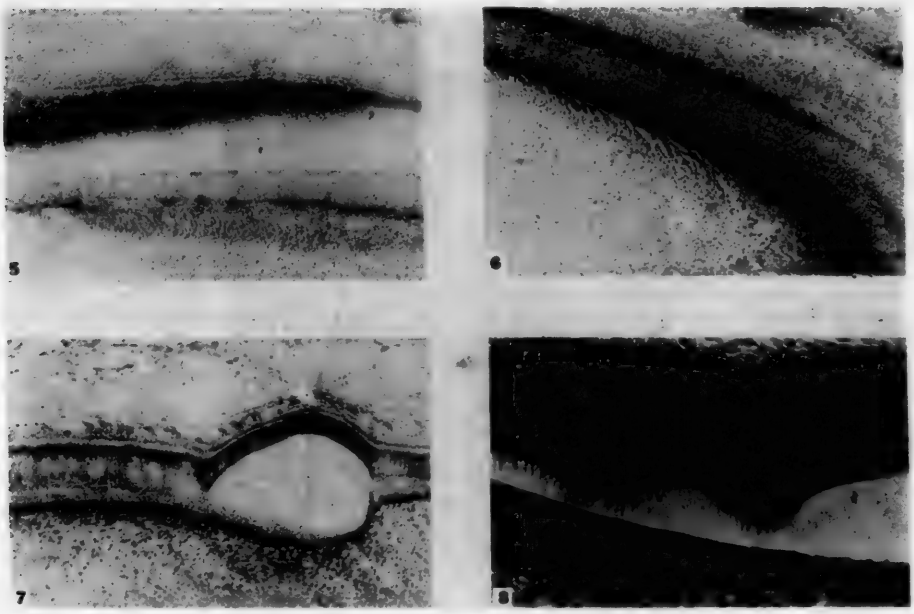

Fig. 5.—Part of first molar, upper jaw from 8-day-old rat, sacrificed two hours after subcutaneous injection of radiosulfate. Inverted autoradiograph of decalcified section (×108; reduced ⅓). There is no difference in enamel picture which is seen at border of tissue and over ameloblasts in area of growth. Reaction over dentin has disappeared with minerals. A minimal reaction is seen over predentin with an apparent accumulation at border of predentin and dentin.

Fig. 6.—Part of lower incisor of 5-day-old rat treated as previously described. At limit of area of growing enamel, which is marked by the cessation of S^{35} incorporation, zone of raised enamel organ can be seen. Inverted autoradiograph (×166; reduced ⅙).

Fig. 7.—Part of lower incisor of 4-day-old hamster treated as previously described. Inverted autoradiograph (×166; reduced ⅓). Incorporation of S^{35} is seen to begin in enamel as the ameloblasts become larger, following area of low and detached epithelium.

Fig. 8.—High-power appearance of cells at onset of enamel growth: ameloblasts and elements of stratum intermedium become larger and clearer. The Tomes's processes appear and elongate. Nuclei of ameloblasts migrate distally. (Mallory's triple stain ×332; reduced ⅙.)

Effect of Hyaluronidase.—Hyaluronidase hydrolysis of similar sections had caused the disappearance of the radiosulfur from dentin, bone, and cartilage after three hours of incubation, but the sulfur bound to new enamel substance as well as that of the mucus glands, mast cells, and inner ear membranes[10] could still be recorded in autoradiographs after six hours of enzyme treatment.

Alkaline Phosphatase.—The histochemical localization of alkaline phosphatase activity has demonstrated a parallel with the previously mentioned areas of S^{35} fixation during the growth phase. In the young teeth, the enzyme was found to be abundantly distributed over the enamel organ and dental pulp as previously described.[16, 20] In the first molar of the 12-day-old rat (Fig. 4), it had been localized only in the cervical portion of the enamel organ, in the stratum intermedium overlying the small group of ameloblasts concerned with S^{35} incorporation.

Toluidine Blue Staining.—Sections of the present material stained with toluidine blue have shown metachromasia in dentin, predentin, bone, cartilage, and other soft tissues where S^{35} had been found incorporated.[19] No metachromasia was detected, however, in any part of enamel or in the ameloblasts.

Fig. 9.—Part of first molar, upper jaw, from 12-day-old rat, sacrificed two hours after tracer injection of radiosulfate. Coated autoradiograph of ground section (×104; reduced ⅓). There is strong reaction over proximal portion of dentin and minor one at dentinoenamel junction, with diffuse picture from this second area upward into enamel. There is also reaction over adjacent alveolar bone. Black spots of different sizes irregularly distributed represent embedded particles of grinding material.

Ferric-Ferricyanide-Basic Fuchsin Staining.—When the technique of Chèvremont and Frédéric[18] was applied, the distal portion of young enamel stained blue. The hairs and the keratinized portion of the skin gave the same reaction. More mature enamel in the incisor stained with less intensity and eventually not at all. When the red basic fuchsin counterstain was applied, the surface portion of enamel stained red in young, forming enamel while the underlying portion stained blue and purple. In more advanced regions, the purple color changed to red again, then to pink, and finally no staining could

be obtained. *Routine staining* with Mallory's connective tissue stain or with hematoxylin-phloxine B-orange G, a modification of the Masson trichrome, has revealed that the area of radiosulfate incorporation at the surface of enamel, like cartilage, bone, and dentin, stained blue with the first dye combination and orange with the second, in contrast to underlying matrix, which stained brilliantly red,[13, 21] like the keratinized portion of the epidermis.

DISCUSSION

The autoradiographs of animals sacrificed one or two hours after the introduction of radiosulfur indicate that SO_4 is rapidly picked up by the ameloblasts and incorporated into an organic substance retained after histologic treatment. The substance is also secreted rapidly and deposited as new enamel or at least part of the new enamel. Some of this material diffuses then into the underlying older enamel (Figs. 3 and 5), but most of it preserves its original relative position as more enamel is laid on top of it. This is well shown by autoradiographs of the forty-eight-hour stage (Fig. 3). Autoradiographs of animals sacrificed later show that the material containing S^{35} seems to disappear progressively from the enamel tissue.

On the other hand, the early illustrations (Figs. 1, 2, 5, 6, and 7) indicate that only the ameloblasts of large type are capable of the operation of S^{35} synthesis and secretion. These observations are in accordance with the theory of a multiphasic enamel organ, and they demonstrate the extent of the secretory phase, which begins with the growth of the cells and their processes (Figs. 7 and 8) and terminates rather abruptly with the decrease in size of the ameloblasts (Figs. 1 and 2). Oftentimes the end of the secretory phase is marked by a short portion of detached epithelium (Fig. 6), already described by Marsland.[3]

The production of alkaline phosphatase in the enamel organ is apparently parallel to the secretory activity of the ameloblasts (Fig. 4). This fact indicates a relationship between alkaline phosphatase activity and growth of the enamel.

The product of secretion of the ameloblasts containing some incorporated sulfate is acid fast (Fig. 5) and apparently hyaluronidase resistant. It does not stain metachromatically, at least under present conditions.

What is this substance? If it is a polysaccharide, it is probably of a type different from that of dentin, bone, and cartilage, which can undergo hyaluronidase hydrolysis under comparable conditions, and possibly similar to that of mucous glands, which are also hyaluronidase resistant.

On the other hand, the rapid incorporation of SO_4 points to an organic combination of sulfuric acid, such as chondroitin or mucoitin sulfuric acid, and thus to a mucoprotein, as described by Pincus.[8, 9]

It is difficult to explain the concentration of radiosulfur seen at the dentinoenamel junction of the older tooth during the maturation phase (Fig. 9). There is no relationship this time with the enamel organ, and the channel of penetration could possibly be the dentinal tubules. A large concentration of

S^{35} is found in the proximal portion of the dentin and represents most likely the polysaccharides demonstrated by metachromatic staining. It is tempting to assume that the radiosulfur deposited in the enamel during the maturation phase, and apparently coming from the inside of the tooth, would be representative of nonprotein-bound polysaccharides, such as described by Wislocki and Sognnaes.[10] These authors stated that in the rat's incisor, only the unerupted middle portion exhibits metachromasia and that "in contrast to the middle portion . . . the innermost and youngest enamel does not stain metachromatically."

Since all our material of this age was embedded in polyester resin and ground, no toluidine blue staining could be carried out.

What about keratin? There is nothing in the present work which points to the formation of keratin. It is possible, but unlikely, that the mucoprotein may be a precursor of keratin. In comparable autoradiographs of skin,[19] no radiosulfate has been detected in the keratin-forming bucal mucosa or skin epidermis at any time. On the other hand, sulfydryl groups were demonstrated histochemically in our material, which is in accordance with the description of Wislocki and Sognnaes.[10] The addition of basic fuchsin to the Turnbull blue picture of the Chèvremont and Frédéric reaction seems to indicate that the basophilic mucoprotein is formed first, and then keratin appears.

How is this organic composition of enamel related to mineralization? The previous autoradiographic mapping of P^{32} and Ca^{45},[12, 13] as well as the histochemical demonstrations of enamel minerals by the silver nitrate technic or polarized light microscopy, have demonstrated that mineral deposition takes place initially in the portion of enamel containing keratin. It is quite possible to assume that the more immature matrix rich in mucoprotein would present a condition of acidity incompatible with mineral deposition. A layer of nonmineralizable substance would then be secreted at the surface of the enamel throughout the period of growth. When the keratin and then the polysaccharides similar to those of dentin and bone appear, the acidity would decrease and mineralization would occur. Logan,[22] Hass,[23] and Sylven[24] have already postulated a similar theory for the mineralization of cartilage, whereby the chondroitin sulfuric acid content would decrease prior to mineral deposition.

On the other hand, assuming that the metachromatic sulfated polysaccharide apparently coming from the inside might be chondroitin sulfate, the role of this substance as premineralization cation binder,[25-27] the "local factor" of Sobel, is recalled.

SUMMARY

Radiosulfate administered subcutaneously to young rats and hamsters has been detected autoradiographically in the ameloblasts and new enamel during the period of growth. It seems to be synthetized into an acid sulfomucoprotein which appears in the matrix before keratin and disappears progressively.

This substance is acid fast and hyaluronidase resistant. When present in large amounts, it may be an inhibitor of mineralization. Its formation and secretion are concomitant with alkaline phosphatase activity.

During the maturation period of the enamel, a concentration of S^{35} appears at the dentinoenamel junction and diffuses upward. It is probably coming from the dentin, and it might represent an invasion of sulfopolysaccharides favoring mineralization.

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TIME STUDIES OF THE PENETRATION OF EXTRACTED HUMAN TEETH BY RADIOACTIVE NICOTINAMIDE, UREA, THIOUREA, AND ACETAMIDE

II. Penetration of Dentin from the Pulp Chamber

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THE testing of the penetrability of dentin with substances more like those normally occurring in the mouth is made possible by the availability of the isotope-labeled compounds nicotinamide, urea, thiourea, and acetamide (Murray, Foreman, and Langham 1947, Murray and Ronzio 1949, Bills and Ronzio 1950). As has been pointed out by Bodecker and Lefkowitz (1946), these compounds are very useful in permeability studies. They do not ionize readily and thus are not influenced as much in their penetration by the presence of electric charges as are inorganic compounds (Klein 1932). A diffuse and unusually rapid penetration of these compounds has been observed in enamel (Wainwright and Lemoine 1950, Wainwright 1954).

Twenty-one unfixed and five formol-fixed extracted human teeth were embedded in Selectron (Wainwright 1949B). The pulp chambers were opened, pulp tissue removed, and the teeth immersed in distilled water at 5° C. until used. At the time of the experiment the tooth was dried with air blast and the pulp chamber filled with the radioactive solution. The solutions were adjusted so that the activity was approximately 1 mc. per milliliter. The specific activities of the compounds were: nicotinamide, 35 μc per milligram; urea, 55.6 μc per milligram; thiourea, 4 μc per milligram; acetamide, 50 μc per milligram. The tooth was stored in a humid atmosphere for the times noted in the tables. Upon removal from the humidifier, part of the excess solution was recovered by washing the pulp chamber with a stream of approximately 15 ml. distilled water within thirty seconds. The sections were cut and surfaced under oil. Survey radioautographs (Wainwright 1949A) were exposed for a constant time of four days.

C^{14}-Nicotinamide.—The distances penetrated from the pulp chamber in ten specimens in periods from eighteen minutes to two days are listed in Table I. It is surprising to find that within eighteen minutes nicotinamide has traveled 3.7 mm. and reached the dentoenamel junction. Every tooth showed at least one area in which the nicotinamide had penetrated 3.5 mm. or more. The five teeth showed a remarkably uniform response in that all were penetrated to the dentoenamel junction.

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TABLE I

DENTIN PENETRATION BY CARBON^{14}-LABELED NICOTINAMIDE

TOOTH		AGE OF PATIENT (YR.)	REASON FOR LOSS	SPECIMEN NO.	RADIOAUTOGRAPH NO.	PENETRATION			
						TIME		MAXIMUM DEPTH (MM.)	PROPORTION DENTIN THICKNESS (%)
NO.	LOCATION					IN HUMIDIFIER	TOTAL*		
390†	UR8	36	Impacted (unerupted)	1081	A834	0	18 min.	3.7	100
				1082	A833		19 min.	2.5	68
391† ‡ §	UR8	35	Paradentosis	1083	A832	18 min.	31 min.	4.1	100
				1084	A825		32 min.	2.5	61
393§ ‖	LR8	32	Impacted (partly erupted)	1085	A837	30 min.	44 min.	3.6	100
				1086	A838		46 min.	3.4	100
394‖	LL8	20	Impacted (partly erupted)	1087	A835	60 min.	71 min.	4.1	100
				1088	A836		72 min.	3.9	100
395¶	LL8	27	Pericoronitis	1089	A843	2 days	2 days	4.6	100
				1090	A844		2 days	4.6	100

*Total time = time in humidifier + time of sectioning, xylene wash, and drying by air blast.

†Extracted and embedded in Selectron one day before experiment.

‡Mesial caries into dentin.

§Occlusal fissure caries of enamel.

‖Extracted two days before experiment; kept in moist gauze one day. Embedded in Selectron one day before experiment.

¶Extracted three days before experiment; kept in moist gauze two days. Embedded in Selectron one day before experiment.

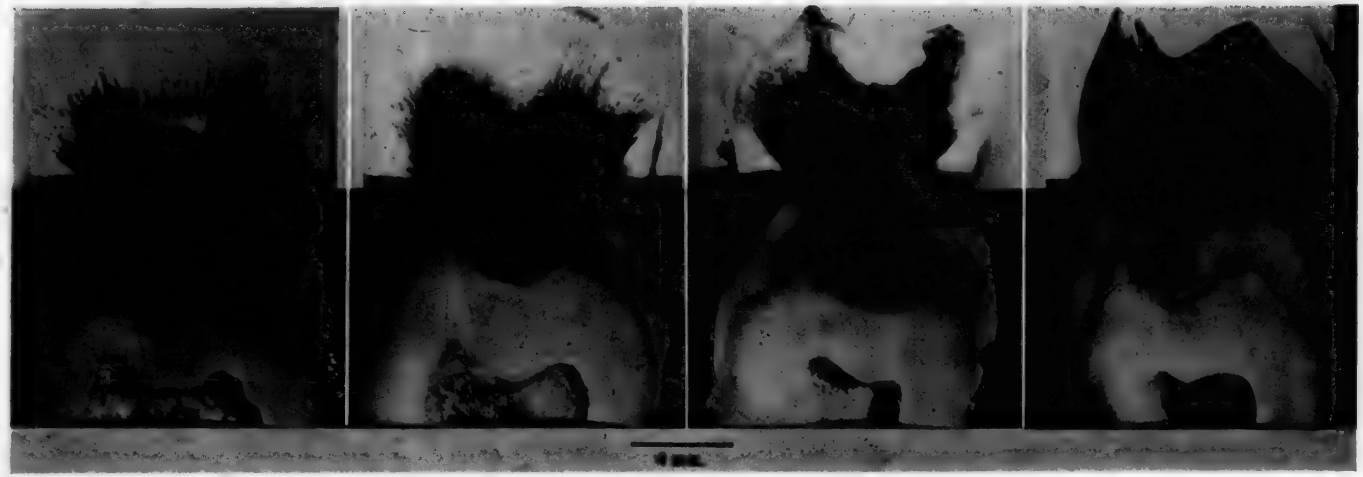
Typical radioautographs and their respective specimens are shown for each time period in Fig. 1. Although the radioactive nicotinamide penetrated to the dentoenamel junction in each of the teeth, the areas of penetration are narrow in the shorter times. In the sixty-minute and two-day radioautographs, the dentin of the entire crown is permeated. A metamorphosed tract under the buccal cusp of the two-day specimen was not penetrated.

C^{14}-Urea.—Shorter periods of time were observed in the experiments with radioactive urea. The values for sixteen specimens from seven unfixed and five formol-fixed teeth are given in Table II. The seven-minute specimen was penetrated a distance of 1.6 mm., nearly halfway through the dentin. Greater proportions of the dentin thickness were penetrated in the eleven-minute subsequent specimens. No difference can be seen due to the effect of formol fixation.

Examples from the series of radioautographs are shown in Fig. 2. Urea shows the same ability to penetrate dentin rapidly. At first the urea follows rather narrow bands to the dentoenamel junction, and in the three-day radioautograph the entire dentin is filled out. The carious portions of the first and last specimens are completely penetrated. A few zones radiating from the pulp chamber are not penetrated, which is especially noticeable in the last three radioautographs.

S^{35}-Thiourea.—Thiourea penetrated very rapidly; in seven minutes it went through 1.4 mm. of dentin, which was over halfway to the dentoenamel junction. The values are listed in Table III. After thirty-eight minutes the entire dentin thickness was penetrated.

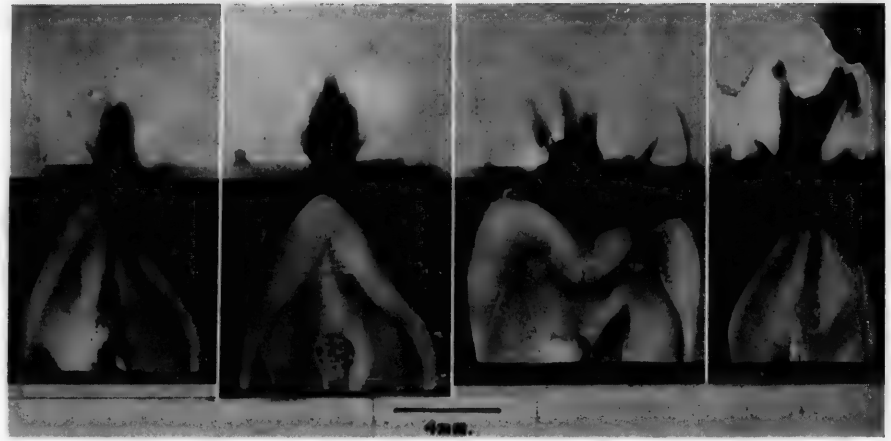
Typical radioautographs are shown in Fig. 3. The teeth of this series are all from the same patient, aged 60 years, and the radioautographs show a decided tendency for penetration along zones rather than uniformly throughout



Time in Humidifier	15 min.	30 min.	60 min.	2 days

Fig. 1.—Penetration of dentin by carbon^{14}-labeled nicotinamide placed in the pulp chamber. Penetration of dentin progresses rapidly. The dentoenamel junction is reached in eighteen minutes, and the entire coronal dentin is permeated in the two-day specimen. The time in the humidifier does not include the time used in sectioning. The total time for each specimen is given in Table I. (15 min.: Spec. 1081, Neg. 15098; R.A.-A834, Neg. 15151. 30 min.: Spec. 1083, Neg. 15114; R.A.-A832, Neg. 15150. 60 min.: Spec. 1087, Neg. 15111; R.A.-A835, Neg. 15135. 2 days: Spec. 1090, Neg. 15113; R.A.-A844, Neg. 15158.)

Fig. 2.—Penetration of dentin by carbon^{14}-labeled urea placed in the pulp chamber. In eleven minutes 1.4 mm. of dentin was penetrated. In the three-day specimen the entire dentin has been filled out to the dentoenamel junction. The time in the humidifier does not include the time used in sectioning. The total time for each specimen is given in Table II. (5 min.: Spec. 957, Neg. 15121; R.A.-A644, Neg. 15158. 15 min.: Spec. 960, Neg. 15122; R.A.-A647, Neg. 15157. 30 min.: Spec. 961, Neg. 15126; R.A.-A648, Neg. 15156. 60 min.: Spec. 1003, Neg. 15125; R.A.-A735, Neg. 15155. 3 days: Spec. 954, Neg. 15108; R.A.-A653, Neg. 15159.)



Time in Humidifier	0 min.	15 min.	30 min.	60 min.

Fig. 3.—Penetration of dentin by sulfur^{35}-labeled thiourea placed in the pulp chamber. In seven minutes 1.4 mm. of dentin was penetrated, and the dentoenamel junction was reached at thirty-eight and sixty-seven minutes. The time in the humidifier does not include the time used in sectioning. The total time for each specimen is given in Table III. (0 min.: Spec. 1103, Neg. 15107; R.A.-A867, Neg. 15149. 15 min.: Spec. 1105, Neg. 15110; R.A.-A866, Neg. 15154. 30 min.: Spec. 1107, Neg. 15109; R.A.-A871, Neg. 15152. 60 min.: Spec. 1109, Neg. 15112; R.A.-A870, Neg. 15153.)

TABLE II

DENTIN PENETRATION BY CARBON^{14}-LABELED UREA

TOOTH		AGE OF PATIENT (YR.)	REASON FOR LOSS	SPECIMEN NO.	RADIOAUTOGRAPH NO.	PENETRATION			
						TIME		MAXIMUM DEPTH (MM.)	PROPORTION OF DENTIN THICKNESS (%)
NO.	LOCATION					IN HUMIDIFIER	TOTAL*		
348†	UL8	26	Caries‡	997	A738	0	7 min.	1.6	44
289§	UL8	23	Pericoronitis‖	955	A642	0	9 min.	0.7	28
				956	A643		9 min.	1.1	44
290§	UR8	24	Malposed¶	957	A644	5 min.	11 min.	1.4	67
				958	A645		11 min.	1.4	40
349†	UR5	19	Pulpitis#	999	A752	10 min.	14 min.	3.6	100
291§**	LL3	32	Paradentosis	960	A647	10 min.	19 min.	1.9	58
292§**	UR3	32	Paradentosis	961	A648	30 min.	36 min.	3.8	100
350†	UR8	24	Malposed	1002	A739	34 min.	42 min.	3.2	100
351†	LR5	31	Caries††	1003	A735	60 min.	64 min.	3.6	100
293§**	UL1	32	Paradentosis	963	A650	60 min.	67 min.	3.2	72
				964	A651		67 min.	2.7	61
286†	LR1	38	Paradentosis	950	A639	1 day	1 day	4.1	100
287†	LL4	26	Caries‡‡	951	A640	2 days	2 days	3.7	100
288†	UL6	32	Caries, paradentosis	953	A652	3 days	3 days	4.3	100
				954	A653	3 days		4.1	100

*Total time = time in humidifier + time of sectioning, xylene wash, and drying by air blast.

†Extracted and embedded in Selectron one day before experiment.

‡Occlusal caries into dentin.

§Formol fixed for three months before experiment and embedded in Selectron.

‖White opacity of enamel surface on distal part of occlusal surface and distal surface.

¶Mesial caries; occlusal amalgam with cement base.

#Disto-occlusal amalgam with cement base.

**From patient P. S.

††Distal caries into dentin.

‡‡Mesial and distal caries.

TABLE III

DENTIN PENETRATION BY SULFUR^{35}-LABELED THIOUREA

TOOTH*		RADIOAUTOGRAPH NO.	PENETRATION			
			TIME		MAXIMUM DEPTH (MM.)	PROPORTION OF DENTIN THICKNESS (%)
SPECIMEN NO.	LOCATION		IN HUMIDIFIER	TOTAL†		
1103	UR1	A867	0	7 min.	1.4	67
1105	UR3	A866	10 min.	17 min.	1.4	42
1107‡	UR5	A871	30 min.	38 min.	2.1	100
1109	LR1	A870	60 min.	67 min.	2.1	100

*Teeth from Patient L. A. G., a 60-year-old woman. Extracted because of paradentosis. Extracted and embedded in Selectron three days before experiment; stored in distilled water two days at 5° C.

†Total time = time in humidifier + time of sectioning, xylene wash, and drying by air blast.

‡Mesio-occlusal amalgam filling.

the entire dentin of the crown. This tendency, although less pronounced than that reported for dyes, may indicate an age change in the dentin which effects the penetration of small molecules as well as large (Bodecker and Lefkowitz 1946, Berggren 1947). Urea penetration is much more rapid than that of dyes, however, and progresses with time, as illustrated, for periods from seven minutes to sixty-seven minutes.

TABLE IV

DENTIN PENETRATION BY CARBON^{14}-LABELED ACETAMIDE PLACED IN THE PULP CHAMBER

TOOTH		AGE OF PATIENT (YR.)	REASON FOR LOSS	SPECIMEN NO.	RADIO-AUTOGRAPH NO.	PENETRATION					
						TIME		DENTIN		ENAMEL	
NO.	LOCATION					IN HUMIDIFIER	TOTAL*	MAXIMUM DEPTH	PROPORTION OF DENTIN THICKNESS	MAXIMUM DEPTH	PROPORTION OF ENAMEL THICKNESS
457	UR1	30	Paradentosis	1300	B158	0	6 min.	1.1†	35†	–	–
452‡	UR5	37§	Paradentosis	1302	B153	15 min.	22 min.	4.1	100	2.1	100
460‖	UR7	29¶	Paradentosis	1303	B154	30 min.	34 min.	3.7	100	1.6	100
				1304	B155		32 min.	3.7	100	2.6	100
461‖	UR8	29¶	Paradentosis	1305	B162	60 min.	63 min.	3.2	100	2.1	100
				1306	B163		64 min.	3.9	100	2.2	100
454#	UR7	37§	Paradentosis	1307	B173	3 days	3 days	2.0	100	4.1	100
				1308	B180			3.2	100	2.9	100

*Total time = time in humidifier + time of sectioning, xylene wash, and drying by air blast.
†Weak diffuse penetration of all dentin.
‡Mesial caries into dentin. Fissure caries.
§Patient O. C.
‖Occlusal amalgam filling with cement base and silver-nitrated carious dentin.
¶Patient L. P.
#Occlusal amalgam filling Distal caries into dentin.

C^{14}-Acetamide.—Five unfixed teeth, extracted and embedded in Selectron the day before, were treated with C^{14}-labeled acetamide for periods of six minutes to three days at 37° C. The distances penetrated in the eight sections are listed in Table IV. One-third (1.1 mm.) of the dentin thickness was penetrated in six minutes. In the next specimen the dentin was penetrated (4.1 mm.), the dentoenamel junction passed, and the enamel faintly penetrated a distance of 2.1 mm. within twenty-two minutes. This rapid penetration was duplicated in the thirty-four-minute, sixty-three-minute, and three-day specimens. When plotted, the curve of penetration approximates a straight line from the pulp chamber at zero time to the dentoenamel junction in about ten minutes and to the surface of the enamel in twenty-five minutes.

The typical radioautographs in Fig. 4 show the comparatively rapid spread of acetamide from the pulp chamber through the dentin into the enamel. Even in the first radioautograph there is a weak diffuse exposure over the entire coronal dentin. The dense area of exposure extends 1.1 mm. into the dentin. The twenty-two-minute radioautograph shows a definite exposure over the entire dentin but is lighter under the cusps. The thirty-two-minute radioautograph shows a weak penetration through the entire thickness of the enamel. The density over the enamel is greater in the sixty-three-minute radioautograph, while after three days, there is a piling up of acetamide at the enamel surface. The three-day radioautograph shows dense penetration in lines suggestive of tufts from the dentoenamel junction into the inner layer of enamel. The middle layers of enamel are densest in lines corresponding to the bands of Schreger. The outer portions of the enamel are less densely penetrated.

DISCUSSION

Since dentin contains tubules which extend from the pulp to the dentoenamel junction and normally are not closed with calcified material, it is not surprising that relatively large particles or molecules, such as ink and dyes, penetrate human dentin.

Experiments on human teeth have usually been conducted for the purpose of studying enamel penetration, and the times have extended for many days. Bodecker and Lefkowitz (1937) observed the in vivo passage of dyes through the dentin of human teeth after experimental periods of seven to forty days. Berggren (1947) sealed dyes in human teeth for periods such as nine days. Bartelstone (1950) has investigated in vivo penetration of I^{131} for periods of twelve hours. (See review by Leicester [1949].) It is surprising, however, to find that an extracted human tooth can be penetrated to the extent of 4.1 mm. of dentin plus 2.1 mm. of enamel in twenty-two minutes. It would be most valuable to know what the performance of these compounds would be in vivo. Bodecker and Lefkowitz (1937) emphasized the differences between experiments performed in vivo and on extracted teeth. They note that "the removal of the dental pulp *temporarily* increases the permeability of the dental tissue. . . ." With the advent of labeled compounds of a type which would normally occur in the oral environment, it is possible to design experiments to determine the rates of physiologic changes in the teeth.

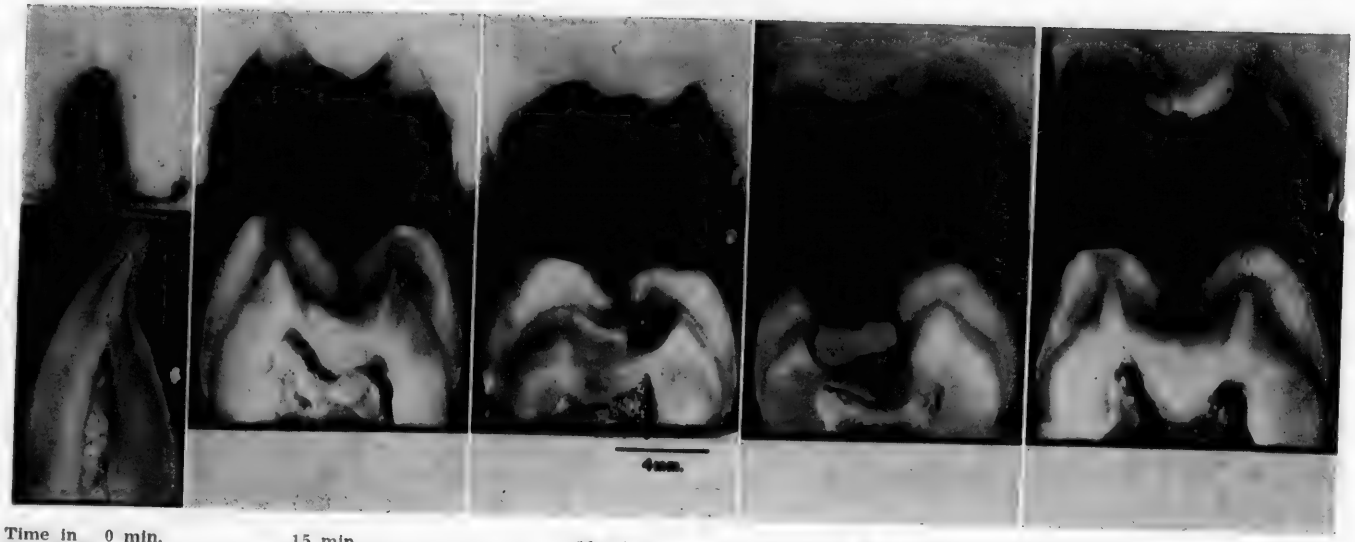


Fig. 4.—Penetration of dentin by carbon^{11}-labeled acetamide placed in the pulp chamber. In six minutes there is a dense penetration through 1.1 mm. of dentin and a weak diffuse penetration of all dentin. At twenty-two minutes there is a strong penetration throughout the dentin, while by thirty-two minutes, penetration has progressed faintly throughout the enamel. After sixty minutes there is a moderate density over the entire enamel. After three days there is a dense penetration to the surface of the intact enamel. The time in the humidifier does not include the time used in sectioning. The total time for each specimen is given in Table IV. (0 min.: Spec. 1300, Neg. 15271; R.A.-B158, Neg. 15165. 15 min.: Spec. 1302, Neg. 15258; R.A.-B153, Neg. 15176. 30 min.: Spec. 1304, Neg. 15259; R.A.-B155, Neg. 15168. 60 min.: Spec. 1305, Neg. 15253; R.A.-B162, Neg. 15174. 3 days: Spec. 1307, Neg. 15261; R.A.-B180, Neg. 15252.)

At present it can only be said that pathways exist for many types of profoundly important processes heretofore considered as being of academic interest only. New and precise tools are at hand for the study of the ways in which vital processes use these pathways.

SUMMARY

1. Isotope-labeled solutions of nicotinamide, urea, thiourea, and acetamide were tested for their ability to penetrate dentin by placing them in the cleaned pulp chambers of extracted human teeth. Twenty-one unfixed and five formol-fixed teeth were treated, stored in a humid atmosphere, and, after periods of six minutes to three days, were sectioned under oil and examined radioautographically.

2. C^{14}-nicotinamide, C^{14}-urea, S^{35}-thiourea, and C^{14}-acetamide penetrated dentin rapidly. Each compound penetrated through approximately 3.5 mm. of dentin to reach the dentoenamel junction in about twenty minutes. Acetamide penetrated through the dentin (4.1 mm.) and faintly through the enamel (2.1 mm.) within twenty-two minutes at 37° C.

The suggestions of Dr. Wright Langham, leader, Bio-Medical Research Group, Dr. Thomas L. Shipman, leader, Health Division, and Dr. John H. Manley, technical associate director, Los Alamos Scientific Laboratory, have been essential to the conduct of the investigation. Grateful acknowledgment is made to Dr. Anthony R. Ronzio, Arthur Murray, III, and C. W. Bills, who prepared the isotope-labeled compounds, and to Miss Agnes Williams who prepared the radioautographs. Teeth were supplied through the cooperation of the Dental Staff of the Los Alamos Medical Center: Drs. Melvin Morrow, director; Nathan Peretzman, Howard Belgorod, F. A. Lemoine, I. R. Quijada, Harold Barrie, and Charles Paraskis.

The C^{14} and S^{35} were obtained on allocation from the Istotopes Division of the United States Atomic Energy Commission.

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A STUDY OF THE ULTRASTRUCTURE OF CHALKY WHITE ENAMEL

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IT HAS been previously reported[15] that topically applied fluorides are distributed unevenly over the surface of the crowns of teeth. Those areas which showed maximal uptake correspond to either chalky white or brown-stained surface imperfections. No single method of photography was able to bring out all these areas, but ultraviolet, infrared, or soft roentgen-ray photography were able to make them visible to the extent that they could be observed in sections of teeth. If such areas are responsible for the uptake of fluorides by teeth, a more detailed knowledge of them is desirable. An explanation of why these areas acquire more fluoride ions than other parts of the tooth would be a preliminary step in such an inquiry. Information of such nature may be gathered with the use of the polarizing microscope. This instrument is of value in depicting the submicroscopic morphology of tissues. With a few exceptions,[4, 7] reports of investigations with polarization microscopy have been confined to the German literature. Most of these studies have dealt with the developing enamel. However, certain fundamental interpretations appeared in these publications which have a broad application to the other fields of study.

The basis of polarization microscopy is an optical property known as birefringence. In biologic specimens this property, also called anisotropy, may take two forms: Intrinsic or "Eigen" birefringence, which is a function of the asymmetrical arrangement of the ions, molecules, or bonds in a structure[9] and is constant as long as the physical and chemical integrity of the material is unchanged, and form birefringence, which is exhibited in materials composed of particles of very small size which are alternately arranged with spaces of similar magnitude. One necessary property of the particles and spaces is that one of their dimensions must be smaller than the wave length of visible light.[5] Not only are there two components (particles and spaces) necessary to produce form birefringence, but the magnitude of this property depends on the difference of refractive indices of these same two elements. By altering the refractive index of either the particles or spaces, the amount of form birefringence can be changed. Such a situation may be brought about by filling the spaces with liquids of different refractive indices. Each newly added agent will alter the form birefringence to a degree which is proportional to the change of the ratio of particle refractive index to surrounding medium refractive index. In order for the various fluids to fill the submicroscopic spaces, there must be an imbibition of the fluid by the specimen.

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Form and intrinsic birefringence may exist independently or together in a given specimen. They may be identified and measured separately, however, by virtue of the immutability of intrinsic birefringence and the predictable variability of form birefringence.

Developing enamel has been shown to have a mixture of intrinsic and form birefringence. The latter is produced by the spaces which exist in incomplete enamel and which are gradually filled by the calcification process. Completion of calcification coincides with the disappearance of form birefringence. The intrinsic birefringence of the enamel particles per se is present throughout the process but is masked in the early stages of enamel formation. As the form birefringence decreases because of filling of the spaces by the calcification process, the intrinsic* birefringence of the enamel particles emerges. The optical changes which coincide with the previously mentioned chronicle of events may best be described with the modifying terms "positive" and "negative." Birefringence refers to the fact that the amount of slowing up of light waves (retardation) in a medium varies according to the plane of direction of the light through the material. If the greatest retardation is parallel to a standard plane of reference through the material (optical axis), then the birefringence is said to be positive. If the plane of greatest retardation is perpendicular to the optical axis, negative birefringence exists. Fully calcified enamel has negative intrinsic birefringence.[7] Slightly calcified enamel has positive form birefringence.* Thus, at the oldest or dentinal end of a forming enamel rod the birefringence may be negative, while at the peripheral end it is positive. Between these two, a zone of isotropy can be found, which is the result of canceling out of the positive and negative forms.

The importance of the aforementioned interpretation, which was originally made by Schmidt and is cited by Harders-Steinhauser and Leicester,[8, 13] lies in the concept of the existence of spaces in structures which exihibit form birefringence.

Since submicroscopic spaces in enamel can be demonstrated, their existence may have a bearing on the ability of certain parts of the tooth to take up topically applied fluorides. If the white spots of the sections which showed selective uptake of F^{18} could be shown to exihibit form birefringence, it would demonstrate that spaces do exist in this type of enamel and that they are capable of imbibing fluids at a rapid rate.

METHODS

Twenty-five human tooth sections having chalky white surface discolorations which selectively retained topically applied F^{18} after washing with water[15] were ground by hand to a thickness of about 100 μ. They were then examined under a polarizing microscope, and the sign of birefringence was determined with the aid of a selenite plate. Each section was then immersed for five minutes in a solution of known refractive index, after which it was placed on a slide with a few drops of the same solution beneath a cover slip.

*Positive form birefringence has been shown mathematically to be equivalent to rodlets and spaces oriented parallel to the long axis of the structure. Negative form birefringence would be equivalent to platelets or discs and spaces the long dimension of which is perpendicular to the long axis.

Estimations of the retardation produced by the white spots and by adjacent normal enamel were made by the interference color chart of Newton.[9] The orientation in each case was such that the long axes of the enamel rods under observation were as parallel as possible to the slow axis of the instrument. This was repeated for each section in each of fourteen liquids with refractive indices from 1.40 to 1.77. The specimens were blotted dry of each reagent before proceeding with the next liquid. The section thickness was then determined by direct measurement with a micrometer gauge accurate to 10 μ. The amount of retardation expressed as millimicrons was divided by the thickness of the section expressed in the same units. This gave an approximate value of birefringence for each section. The entire chalky white area did not exhibit a change in birefringence as the liquids were changed, but certain portions of it did. These were used for the determinations. To make the measurements as reproducible as possible, the same area of sound and chalky white enamel was compared for each liquid.

FINDINGS

Examination of the chalky white region of the enamel revealed that its birefringence was different from that of the normal-appearing adjacent structure. In general, the chalky areas were characterized by a tendency to exhibit a birefringence more nearly isotropic or positive than that of the neighboring enamel. The range of variation included some chalky enamel which was plainly positive and some which was merely less negative than the usual state. Distributed throughout these optically altered areas were islands whose color revealed them to be more markedly birefringent in the positive direction than the rest of the chalky enamel. These were multiple, small in size, and were located in a random manner. In a given section, all of them did not exhibit the same degree of birefringence, but generally they indicated the most altered birefringence anywhere in the section. These same isolated areas were the only ones which underwent a color change when immersion of the specimen was attempted.

TABLE I

VARIATION OF MAGNITUDE AND SIGN OF BIREFRINGENCE OF CHALKY ENAMEL IN DIFFERENT IMMERSION MEDIA

NAME OF LIQUID	REFRACTIVE INDEX	BIREFRINGENCE
Amyl alcohol	1.40	+0.00110
Ethylene chloride	1.44	+0.00025
Carbon tetrachloride	1.46	+0.00020
Xylene	1.50	None
Oil of cloves	1.53	-0.00025
Monobromphenol	1.58	-0.00070
Oil of cassia	1.60	-0.00110
Cinnamic aldehyde	1.61	-0.00130
Carbon bisulfide	1.62	-0.00110
-Monochlornaphthalene	1.63	-0.00130
-Bromonaphthalene and -chloronaphthalene	1.64	-0.00085
Methylene iodide	1.74	-0.00025
Methylene iodide with sulfur	1.76	-0.00014
	1.77	-0.00011

Table I presents the values of birefringence found for the islands in a chalky area of a representative section in each of the liquids used. It may be seen that the birefringence changed from positive to isotropy as liquids having refractive indices from 1.40 to 1.50 were used for immersion of the specimens. As the refractive index was increased, negative birefringence appeared. Maximal negative birefringence occurs for media with refractive indices of about 1.62. Increasing the refractive index beyond this results in diminished negative birefringence. Fig. 1 is a representation of the data in Table I. It may be seen that the curve is parabolic in shape with its extremum corresponding to a refractive index of about 1.62.

Normal enamel exhibited no change of birefringence in any of the liquids used.

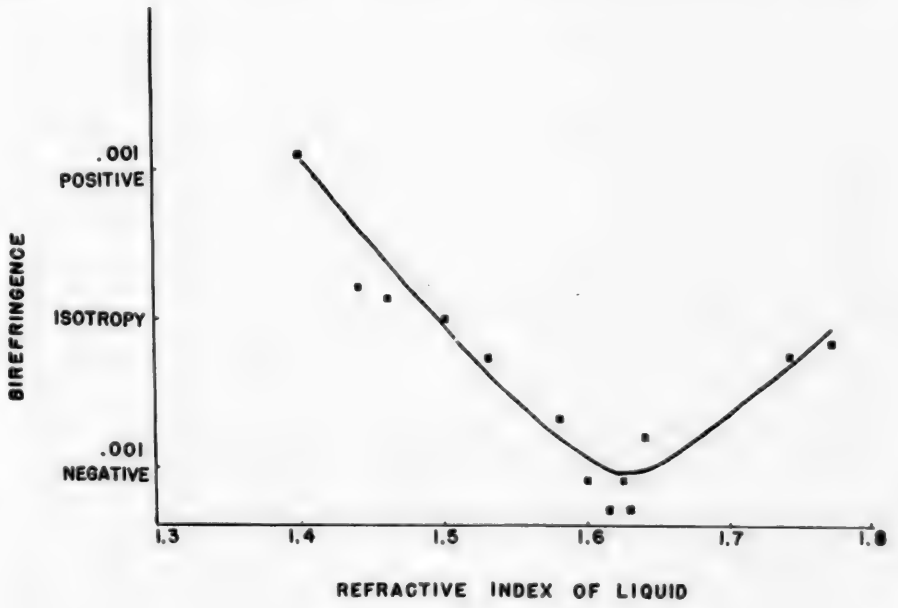

Fig. 1.

DISCUSSION

The "U" shape of the curve presented in Fig. 1 indicates that form birefringence may be found in certain areas of the chalky spots which showed a preferential retention of F^{18}. This means that spaces exist in these areas which can imbibe fluids to the degree that the form birefringence of the specimen may be altered. The closer that the refractive index of the imbibed fluid is to the refractive index of the particles, the smaller is the form birefringence. At a refractive index of about 1.62, form birefringence is obliterated, and the remainder may be regarded as the true or intrinsic birefringence of the particles. It is obvious that it does not matter whether the medium

has a greater or lesser refractive index than the particles it surrounds; the form birefringence is dependent only on the difference between these two values.

Normal enamel exhibited no islands of form birefringence, the various media having had no effect on its optical properties. The negative birefringence of these portions of enamel is not subject to imbibition phenomena and may be postulated to have either no or much smaller spaces in its ultrastructure than are found in chalky enamel of similar locations.

The presence of imbibition phenomena in a portion of the chalky enamel suggests that its positive birefringence is due to a deficiency of inorganic salts. This is likely since in vitro decalcification of enamel makes the phenomenon of imbibition possible.[10] Some regions of the chalky white area have an altered birefringence but are of a nature which exhibits no imbibition. The evidence in the literature suggests that these have a deficiency of organic material, inasmuch as ashing enamel does not produce imbibition.[10]

It is desirable to distinguish between the phenomenon of imbibition and that of permeability of enamel. The chief difference lies in the manner in which these two phenomena are demonstrated. Imbibition is always reported to occur on the basis of changes in optical properties which it brings about. The birefringence or translucency of enamel has been changed whenever imbibition was claimed.[6, 13, 14] Permeability is determined by locating the salt[11, 12] or dye molecule[3] in the enamel. No statements about the optical properties are necessary since only the ion or salt is identified.[1, 17, 18] Furthermore, it is generally believed that the penetration of enamel by molecules and ions is by way of the organic matter.[13] Imbibition, then, implies the presence of liquids in those parts of enamel which influence changes in its optical properties. Permeability, on the other hand, implies channels in the organic component of enamel sufficient only to allow the passage of an ion or molecule.

Bear, Schmitt, and Young[2] cited a formula developed by Wiener which applies to simple ideal systems exhibiting form birefringence. This formula has been modified by these authors to a more usable form.[2] Using this latter formula it can be shown that the slope of a curve such as the one in Fig. 1 is a function of the product of the respective volume fractions of the particles and spaces.[2] If these two components occupied equal volumes, then each volume would be represented by the fraction 0.5. The product of the two fractions would be 0.25. This is the largest value which this number could have, and the curve corresponding to such a situation would have a maximally steep slope. As either of the components is reduced in volume, the product of the two values becomes smaller and the curve becomes flattened. When only one component exists, as in intrinsic birefringence (no spaces), the product is zero and the curve becomes a flat horizontal line. Applying this formula to our data, the product of the two volume fractions is found to be much too small to be applicable to the curve in Fig. 1. This suggests that the simple, ideal conditions of form birefringence assumed in Wiener's formula do not apply here.

SUMMARY AND CONCLUSIONS

1. Chalky white enamel which selectively retains topically applied F^{18} is identifiable under a polarizing microscope by its altered birefringence.

2. Islands within chalky enamel exhibit imbibition of liquids. Positive form birefringence has been identified in these areas. This indicates the presence of submicroscopic spaces at these sites.

3. Enamel which is unaltered in gross or microscopic appearance and does not exhibit preferential retention of topically applied F^{18} shows no areas of form birefringence.

4. The presence of submicroscopic spaces may be related to the selective uptake of fluoride ions by chalky enamel.

I wish to thank Dr. Harry E. Frisbie, of the College of Dentistry, University of California, for his generosity in making the polarizing microscope available to me. I would like to express my appreciation to Drs. Hermann Becks, of the University of California College of Dentistry, and Henry M. Leicester, of the College of Physicians and Surgeons, San Francisco, for their encouragement and advice.

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THE MECHANISM OF HYGROSCOPIC EXPANSION IN DENTAL CASTING INVESTMENTS

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INTRODUCTION

ONE of the fundamental problems in restorative dentistry is the fabrication of cast gold appliances which accurately fit the cavity prepared in a tooth or the space between teeth located anywhere in the dental arches. Two important factors which must be considered in making such castings are the dimensional change of the wax pattern and the contraction or casting shrinkage of gold on cooling from the liquid state to room temperature. At the present time there are two widely used technics that compensate for these shrinkages. Both depend upon expansion of the casting investment.

The older, more universal, technic, often referred to as the "thermal expansion" method, depends upon the presence in the investment of a material or materials which, when heated, will expand in a known manner to enlarge the mold.

The more recently introduced method for increasing the mold size is based on the observation that some types of investment materials, when placed in water during the setting period, expand three to four times their normal setting expansion. The mechanism of this enlargement, commonly termed "hygroscopic expansion," will be discussed.

The phenomenon of hygroscopic expansion was reported by Wilson[1] and was called to the attention of the dental profession by Scheu.[2] Scheu's original observations on the expansion of setting inlay investment upon the addition of water are the basis for the present hygroscopic technic. Scheu noted that all investments expanded hygroscopically and that the amount of expansion depended upon the water-powder ratio and time of immersion. Scheu defined hygroscopic expansion as that expansion occurring when water comes in contact with a mass of setting investment.

Numerous investigators[3-6] have published the results of their findings following Scheu's original observations. Degni[7] stated that the hygroscopic expansion was extremely sensitive to confinement of any kind. Degni also used the colloidal theory for the setting of plaster of Paris to explain the hygroscopic expansion process.

Landgren[8] presented an excellent report on some of the factors affecting hygroscopic expansion. Landgren noted that the water-powder ratio played an important role in determining the amount of expansion. He showed that the thicker the mix, the greater the amount of hygroscopic expansion. Landgren also showed that hygroscopic expansion decreases with an increase in time between spatulation and immersion when immersion is made after the initial set.

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Generally speaking, the investigations mentioned previously have been primarily concerned with measuring the amount of expansion which can be obtained hygroscopically and with determining the factors which affect such expansion. The mechanism of expansion has not been definitely established. It is the purpose of this paper to present a possible explanation of this phenomenon and to report some data supporting the explanation.

MECHANISM OF SETTING AND HYGROSCOPIC EXPANSION

Reactions Involved During the Normal Setting Expansion.—Typical inlay investments contain approximately 30 per cent gypsum (plaster of Paris or hydrocal) and 65 per cent silica, the gypsum serving as a bonding medium for the silica. Plaster of Paris and the closely allied compound, dental stone or hydrocal, are essentially hemihydrates of calcium sulfate, $CaSO_4 \cdot \frac{1}{2} H_2O$. When the hemihydrate is mixed with water, the dihydrate $CaSO_4 \cdot 2 H_2O$, or gypsum, is formed. The solubility of the hemihydrate is 0.65 Gm. per 100 ml. of water and of the dihydrate, 0.21 Gm. per 100 ml. at 23.5° C.[9] Thus, when plaster is mixed with water, a nearly saturated solution of the hemihydrate and a supersaturated solution of the dihydrate are quickly obtained. As the dihydrate is rapidly precipitated from solution, more hemihydrate is dissolved and crystallization continues. This process of solution and crystallization continues until the precipitated crystals have intermeshed so tightly that solidification occurs.

The hydrated gypsum formed in this process has a density of 2.32[10] and a molecular weight of 172, giving a molar volume of 74.2 ml. Values varying from 2.55 to 2.75 have been reported for the density of the hemihydrate.[9, 10] Since the hemihydrate has a molecular weight of 145, this gives a molar volume of approximately 55 ml. Thus, a volume increase of approximately 35 per cent occurs on hydration. However, if the 27 ml. of water required for this reaction is considered, a net shrinkage of 10 per cent occurs. This shrinkage is not observed in a specimen of setting plaster. A slight expansion always occurs. The explanation of this apparent anomaly is based on the crystalline growth of the precipitated gypsum, which results in a porous structure with a greater apparent volume. Thus, the setting expansion observed in plasters results from crystal growth after and during the period when the mass becomes sufficiently solid to retain a definite form and just prior to the time at which the material attains sufficient strength to resist further deformation.

Apparently the hygroscopic expansion observed in investment materials results from an increased crystal growth over the period of time in which the mass is in a semisolid state and is a continuation of the normal setting expansion. The observations made during this investigation of the effect that inert substances and added water have on the amount of hygroscopic expansion appear to have a direct bearing on this proposed mechanism of continued expansion.

Effect of Inert Substances.—The expansion observed when hydrocal is mixed with water is of the order of 0.04 to 0.06 per cent. Hydrocal cannot be made to exhibit hygroscopic expansion. It was observed, however, that if an inert material was added to the hydrocal prior to mixing with water, the resulting mass invariably expanded hygroscopically. In these experiments the following

inert materials were employed: silica, zinc dust, iron powder, acrylic resin powder, pumice, graphite, silica gel, sulfur, lampblack, and talc. All the resulting mixes exhibited hygroscopic expansion. Some of the materials, particularly those difficult to wet, exhibited less expansion than others. Typical results obtained are shown in Table I.

TABLE I

HYGROSCOPIC EXPANSION OF MIXTURES OF HYDROCAL AND INERT SUBSTANCES

	SETTING EXPANSION	
SUBSTANCE ADDED	INERT SUBSTANCE 20 PER CENT BY WEIGHT (PER CENT)	INERT SUBSTANCE 60 PER CENT BY WEIGHT (PER CENT)
Pumice	0.4	0.75
Graphite	0.7	1.6
Talc	0.8	2.8
Silica gel	3.1	8.1

It appears that the effect of the inert material was to weaken the structure of the mass to such an extent that expansion resulting from crystalline growth could continue for a longer period of time before the mass gained sufficient strength to resist further deformation. Such crystalline thrust or growth is not uncommon in the solidification of metals from the liquid state.[11]

In addition to loosening the structure of the setting investment, the presence of inert materials may allow more efficient penetration of water into the structure of the investment mass.

Effect of Water.—To make an investment expand hygroscopically, additional water must be made available to the investment during a critical period in the hardening of the mass. This need for additional water is difficult to explain, particularly because of the fact that water in excess of that required for complete hydration is always added in making the initial mix. The following explanation for this need of additional water is proposed.

In the setting reaction of investments, crystallization does not occur uniformly throughout the mass. During this nonuniform crystallization, powder particles begin to dissolve and crystals of the hydrate grow on the surface of the undissolved hemihydrate. Further crystalline growth causes water to be retained in the voids in and around the crystallites. Thus, although sufficient water is present initially to hydrate all the hemihydrate, it is temporarily prevented from doing so by this entrapment. Eventually, by a process of diffusion of the water or water vapor, complete hydration will occur. However, the process is slow and the mass will have gained sufficient strength to resist deformation before hydration is completed.

If the investment is placed in water prior to such hardening, the water rapidly penetrates the mass as a result of capillary action. Additional hemihydrate is dissolved, dihydrate is precipitated, and further crystallization continues with sufficient rapidity to expand the semisolid mass. That some such mechanism is in operation is made evident by the rapidity with which water is imbibed by the mass during this critical period and its immediate effect in expanding the mass. This was demonstrated by placing a cylindrical specimen of investment 1.25 inches in diameter and 1.5 inches high under a dial gage. At

the critical period during which hygroscopic expansion occurs most readily, drops of water were applied to various areas over the surface of the specimen by means of a medicine dropper. An immediate increase in length was observed as each drop of water was added.

In other experiments a small amount of water was placed around the base of a specimen. When the setting reaction reached the critical period for hygroscopic expansion to occur, the water was rapidly pulled into the specimen and expansion followed immediately.

Volume Changes During Hydration.—If the proposed mechanism of further crystallization is valid, some of the hemihydrate must remain unhydrated during the period in which the setting mass is most susceptible to hygroscopic expansion. That hemihydrate is present in the mix during this period was demonstrated by an experiment in which the rate of hydration was observed by measuring the decrease in volume of a mixture of investment and water containing a large excess of water. The investment powder was inserted into an apparatus so designed that all air could be pumped out of the powder mass and water added as shown in Fig. 1.

Sufficient water was added to cover the specimen and rise in a graduated capillary tube joined to the specimen chamber. The apparatus, sample, and water were in an air bath maintained at a constant temperature to within ± 0.1° C. The height of the water in the capillary tube was recorded at intervals up to three hours after the water was admitted to the specimen chamber.

The results obtained on several brands of investment and one dental artificial stone are shown in Fig. 2. The data show that shrinkage of the investment-water mixtures continued at a rapid rate for approximately one hour after the investment and water were mixed. This indicates that hemihydrate is present in the mix and that hydration continues during the time that hygroscopic expansion occurs. It is reasonable to believe, therefore, that water added to the investment causes hygroscopic expansion to occur by inciting a more complete hydration while the material is in a semisolid state. It is possible that the water added weakens the structure of the setting investment and thus permits a greater expansion of the investment by the growth or thrust of the precipitating hydrate crystals.

Effect of Retarders and Accelerators.—If hygroscopic expansion is only an extension of setting expansion as a result of sorption of water during a critical period in the setting of gypsum, as proposed, materials which retard the hydration and hardening of gypsum would be expected to lengthen this critical period, thus permitting more sorption of water and causing an increase in the hygroscopic expansion of investments. To test this, cylindrical specimens of an investment to which a retarder had been added were prepared. These cylinders were placed under a dial gage as soon as they possessed sufficient strength to support themselves. Water was then poured into the container surrounding the investment and the amount of expansion was measured on the dial gage. Similar experiments were made on specimens containing no retarder. The increased amount of expansion in specimens containing retarders is shown in Table II. The retarders apparently prevented rapid hydration and allowed greater crystalline growth in a weakened structure.

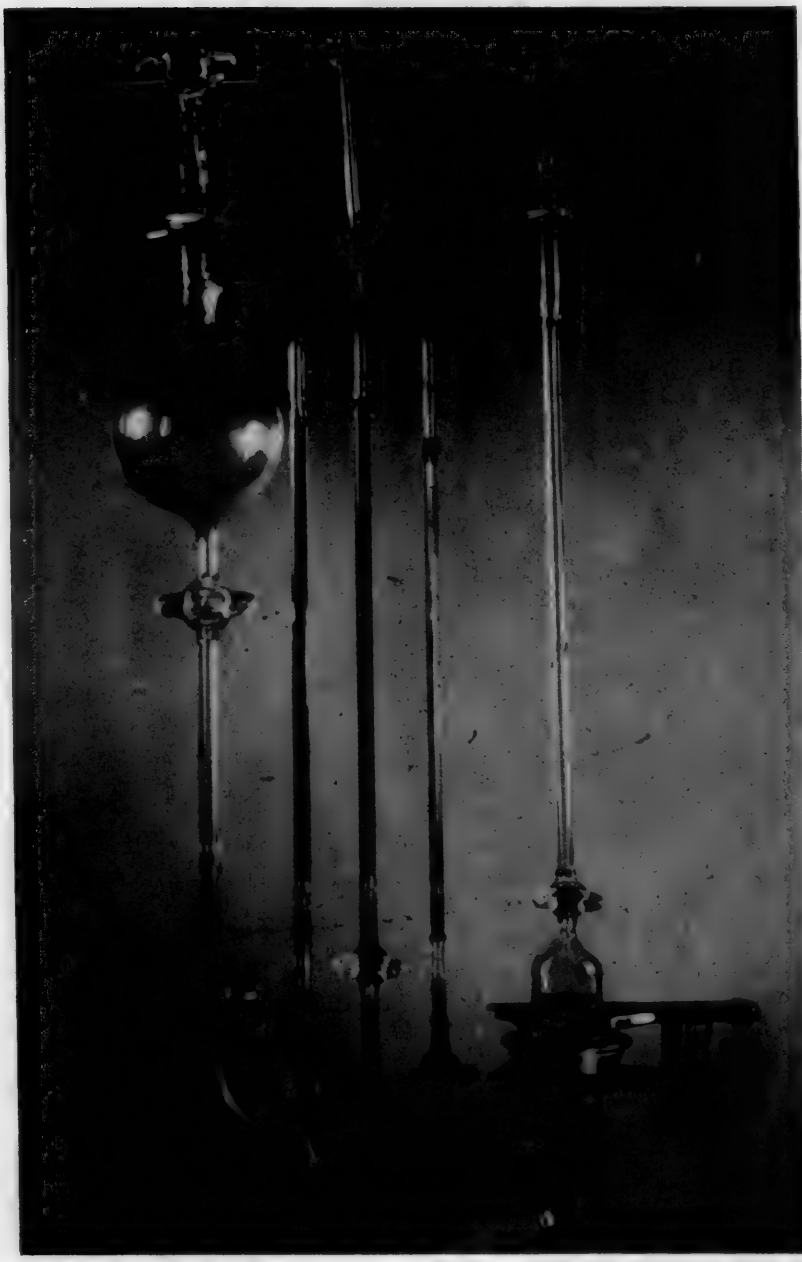

Fig. 1.—Apparatus used to investigate rate of hydration of gypsum in water by measurement of decrease in volume resulting from hydration. Gypsum is placed in specimen chamber at bottom of apparatus. After this chamber has been evacuated, water from spherical reservoir is allowed to flow around specimen. Reservoir is then sealed off, appropriate capillary tube opened, and change in volume is observed during period of hydration of specimen.

TABLE II

EFFECT OF ACCELERATORS AND RETARDERS ON HYGROSCOPIC SETTING EXPANSION OF CRISTOBALITE INVESTMENT

MATERIAL USED	ACTION	SETTING TIME (MIN.)	EXPANSION (PER CENT)
Cristobalite investment + ferrous sulfate, 2 per cent; wet asbestos liner; not immersed	Accelerator	10	0.6
Cristobalite investment + ferrous sulfate, 2 per cent; wet asbestos liner; immersed	Accelerator	10	0.6
Cristobalite investment, wet asbestos liner; not immersed	None	25	1.0
Cristobalite investment; freely expanding; immersed	None	25	1.5
Cristobalite investment + sodium acetate, 4 per cent; wet asbestos liner; not immersed	Retarder	90	1.2
Cristobalite investment + sodium acetate; freely expanding; immersed	Retarder	120	2.2

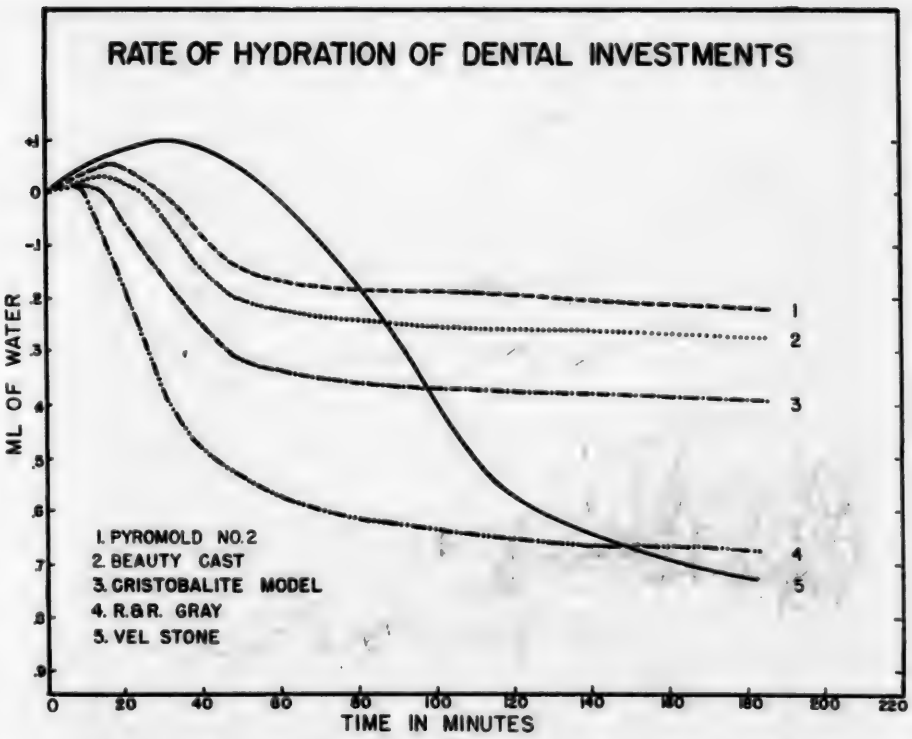

Fig. 2.—Rate of hydration of dental investments and stone as indicated by change in volume of water and investment or stone mixture. Change in volume of mixture is indicated as milliliters of water.

The experiment was repeated, substituting accelerators for retarders. The extreme rapidity of set resulted in a much lower expansion. Probably because of the increased speed of hydration and the rapid interlocking of the crystals into an extremely hard mass in a short period of time, the amount of expansion was not affected by immersion in water.

Production of Hygroscopic Expansion by Silica Gel.—Based on the principles demonstrated in the experiments on the effect of inert substances and water on hygroscopic expansion, an experimental investment was prepared using silica gel previously saturated with water and air-dried, mixed with hydrocal. Specimens of this investment containing 50 per cent hydrocal and 50 per cent silica gel expanded approximately 3 per cent without the addition of water. The amount of expansion of other specimens containing different ratios of hydrocal to silica gel was roughly proportional to the amount of silica gel added. It is postulated that the silica gel, capable of holding up to ninety times its own weight of water, acted both as an inert material and as a source of water for the continued hydration of the hemihydrate.

Although these experimental investments behaved in accordance with the proposed mechanism for hygroscopic expansion, they were not satisfactory for practical use in an inlay technic.* However, this preparation of an investment which will expand hygroscopically without the addition of water suggests the possibility of the development of a satisfactory dental investment based on this principle.

CONCLUSIONS

On the basis of the data presented it is believed that hygroscopic expansion of dental investment is a continuation of the normal setting expansion during the period when the investment is in a semisolid state. This continued expansion is produced by the introduction of additional water which permits further hydration of calcium sulfate at this time. The inert filler present in the investment weakens its structure and thus permits the precipitating hydrate crystals to expand the mass of material.

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*Heating these investments caused shrinkage and reduced strength. Castings made in unheated molds were unsatisfactory, apparently because of the vaporization of water in the investment by the molten gold.

MEASUREMENT OF THE ELECTRIC CONDUCTIVITY OF DENTAL CEMENT

II. Values for Specimens Exposed to Moisture

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INTRODUCTION

A DISCUSSION of the instrumentation, circuit design, necessary calculations, and cell construction which have been developed for accurately measuring the electric conductivity of dental cements has been presented.[1] A few preliminary experiments were summarized to show the resistance pattern upon the aging of the cement specimen. Values were obtained on the electric conductivity of dry specimens of zinc phosphate cement. Furthermore, there was an evaluation of the influence played by open and closed circuits and the direction of current flow, all data correlating resistance as a function of time. In those tests the cement was carefully sealed against loss or gain in moisture. This factor had been willfully controlled in order to eliminate one of the variables associated with the construction of the test cells. It was recognized that a cement base, or the thin layer of cement surrounding a gold inlay, is not so dry as these specimens. The actual amount of moisture or fluid to which the cement is exposed in the cavity preparation is, of course, unknown. Additional tests have now been made, however, with the cement margin exposed to normal saline solution and also to distilled water. It is the purpose of this paper to present these data.

EFFECT OF MOISTURE

A group of cells were constructed similar to those that were used in the previous tests. These cells, however, had the cement margin exposed to distilled water exactly one hour after mixing of the cement. This was accomplished by taking the same type of cell used previously and removing some of the sealing compound so that the margin of the cement was exposed. A typical cell, showing the exposed margin, is seen in Fig. 1. The cell was then placed in a jar containing distilled water. The jar was closed and the leads brought out through the sealing wax. This method prevented evaporation of the water and also any possible contamination from the air. Nine cells were tested for a period of 100 days; the data can be seen in Table I.

The initial reading of the cells was staggered with respect to time in order to see whether the actual reading had any effect on the resistance pattern. It

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was found that it did not. As can be seen from the data, the pattern is one of increasing resistance with time. However, it is to be noted that the magnitude of the resistance is much less for a given period of time when compared to that obtained for the dry specimens. For example, at 768 hours most of the dry specimens had reached 1,000 megohms, while the average value obtained with the exposed margins for the same time interval is only 2.89 megohms. The presence of moisture is apparently a definite factor in controlling the resistance of the cement.

TABLE I

TIME-RESISTANCE CHART: AMALGAM, ZINC PHOSPHATE, COPPER CELLS; CIRCUIT B, MARGIN EXPOSED TO DISTILLED WATER (ELECTROMOTIVE FORCES BUCKING)

TIME OF READING ASSEMBLY (HR.)	CELL NUMBER AND RESISTANCE IN MEGOHMS									
	I	II	III	IV	V	VI	VII	VIII	IX	AVG.
2	0.41									0.41
4	0.34	0.44								0.39
8	0.62	0.52	0.63							0.59
16	0.54	0.79	0.47	0.82						0.63
32	0.65	0.79	0.45	0.67	0.58					0.63
64	1.60	1.21	0.67	1.00	0.64	0.88				1.00
128	1.92	1.89	0.95	1.67	1.11	0.86	1.37			1.40
256	2.22	2.32	1.15	2.18	1.42	1.24	1.91	0.75		1.65
384	3.31	5.14	1.49	2.62	2.09	1.74	2.40	0.88	1.16	2.32
512	3.50	4.14	1.28	2.87	2.34	2.15	1.71	1.20	1.23	2.27
768	4.26	6.04	1.46	3.84	2.99	2.59	1.79	1.66	1.37	2.89
1,200	1.32	4.05	2.36	6.90	2.84	3.26	2.15	1.69	1.52	2.90
2,400	4.88	4.09	1.81	5.99	3.69	1.93	2.38	1.32	1.89	3.11

TABLE II

TIME-RESISTANCE CHART: AMALGAM, ZINC PHOSPHATE, COPPER CELLS (MARGIN EXPOSED TO 1 PER CENT SALINE SOLUTION)

TIME OF READING AFTER ASSEMBLY (HR.)	CELL NUMBER AND RESISTANCE IN MEGOHMS									
	CIRCUIT B CLOSED CIRCUIT					OPEN CIRCUIT EXCEPT WHEN MEASURED				
	I	II	III	IV	AVG.	V	VI	VII	VIII	AVG.
2	0.040	0.040	0.195	0.075	0.088	0.052	0.035	0.220	0.082	0.097
4	0.040	0.040	0.052	0.040	0.043	0.030	0.035	0.052	0.075	0.048
8	0.125	0.145	0.062	0.062	0.098	0.062	0.062	0.065	0.100	0.072
16	0.198	0.160	0.075	0.135	0.141	0.065	0.044	0.052	0.118	0.077
32	0.172	0.150	0.075	0.125	0.131	0.040	0.040	0.025	0.082	0.047
64	0.105	0.090	0.105	0.105	0.101	0.020	0.040	0.035	0.078	0.043
128	0.118	0.150	0.118	0.145	0.133	0.024	0.044	0.062	0.041	0.043
256	0.060	0.062	0.056	0.062	0.060	0.058	0.170	0.058	0.061	0.087
384	0.047	0.082	0.095	0.037	0.065	0.043	0.182	0.062	0.072	0.090
512	0.078	0.092	0.135	0.040	0.086	0.082	0.220	0.082	0.092	0.119
768	0.075	0.102	0.104	0.078	0.090	0.080	0.217	0.070	0.150	0.129
1,872	0.085	0.045	0.042	0.092	0.066	0.099	0.162	0.078	0.061	0.100
2,568	0.060	0.060	0.042	0.060	0.056	0.130	0.220	0.082	0.082	0.129

Another group of cells similar to the previous group was tested, with the exception that the cells were exposed to a 1 per cent saline solution instead of distilled water. The results were similar to those of the distilled water group, although the average resistance was somewhat less in magnitude. Two groups

of cells were run, one under closed-circuit conditions and one under open-circuit conditions. The two groups behaved in a similar manner and showed no appreciable differences. Table II shows the data collected from these cells. These data are summarized in Fig. 2.

Fig. 1.—Conventional cell with exposed margin.

The general comparison between wet and dry cells can be seen in Fig. 3. The resistance pattern for three groups of dry cells (closed-circuit aiding, closed-circuit bucking, and open circuit) is plotted, as well as the resistance of the wet cells when tested under a closed circuit with electromotive forces bucking. The great reduction in resistance when exposed to moisture is obvious. Consequently, in order to evaluate the actual insulating ability of the cement, the amount of moisture present must be taken into consideration. In the mouth, the cement margins of an inlay are exposed to mouth fluids. Likewise, the cement bases under amalgam restorations are subjected to dentinal moisture. The effect of moisture under clinical conditions remains to be studied, but these tests would indicate that the resistance is greatly lowered when fluids are in contact with the cement. What apparently was more than adequate insulating ability in the sealed state may be borderline or inadequate in the moist condition.

The effect of moisture on the conductivity of cement was studied further by an additional series of tests. Four cells, which were constructed in the

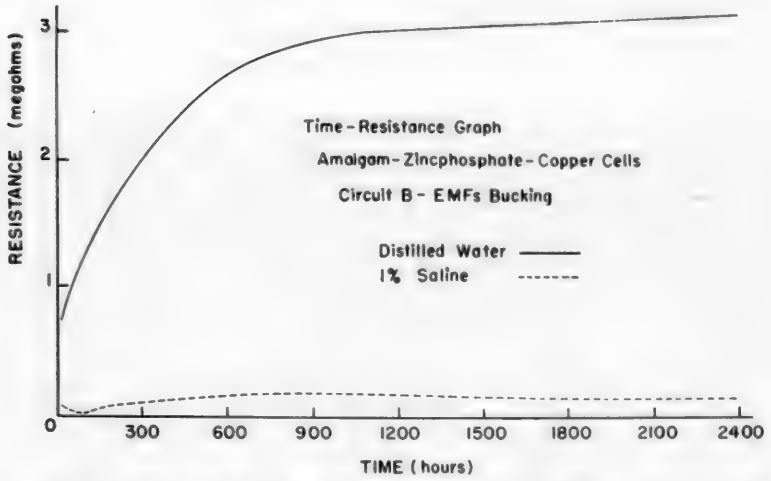

Fig. 2.—Cells with margins exposed to saline solution and to distilled water.

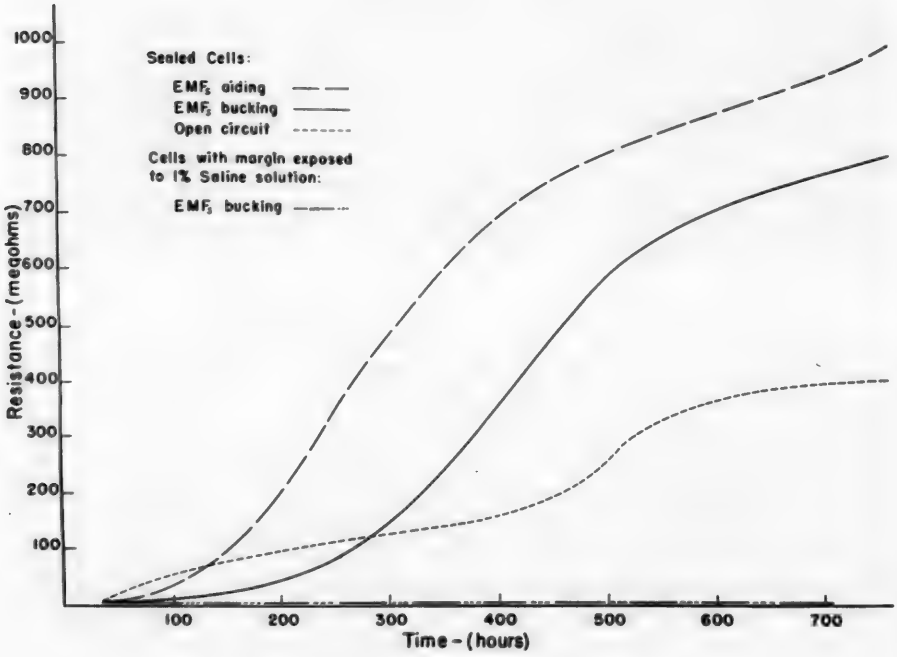

Fig. 3.—Exposed margins versus sealed margins.

same manner as those in previous tests, were used and the resistance recorded as before. All four cells were sealed and run on a closed circuit (Circuit B). The data can be seen in Table III. At the end of 1,200 hours, they had measured values of resistance in excess of 1,000 megohms. These cells then had the cement margins exposed to 1 per cent saline solution for ninety-one hours. It is noted that the resistance of all four decreased markedly after the margins were exposed. At the end of ninety-one hours, all four were removed from the saline solution and again sealed with compound. Two were placed on closed circuit (Circuit A), and two were placed on an open circuit. The resistance at designated time intervals was noted. At the end of 164 hours, the margins were again exposed to 1 per cent saline solution for fifty-eight hours. At that time they were sealed again, but the two which previously had

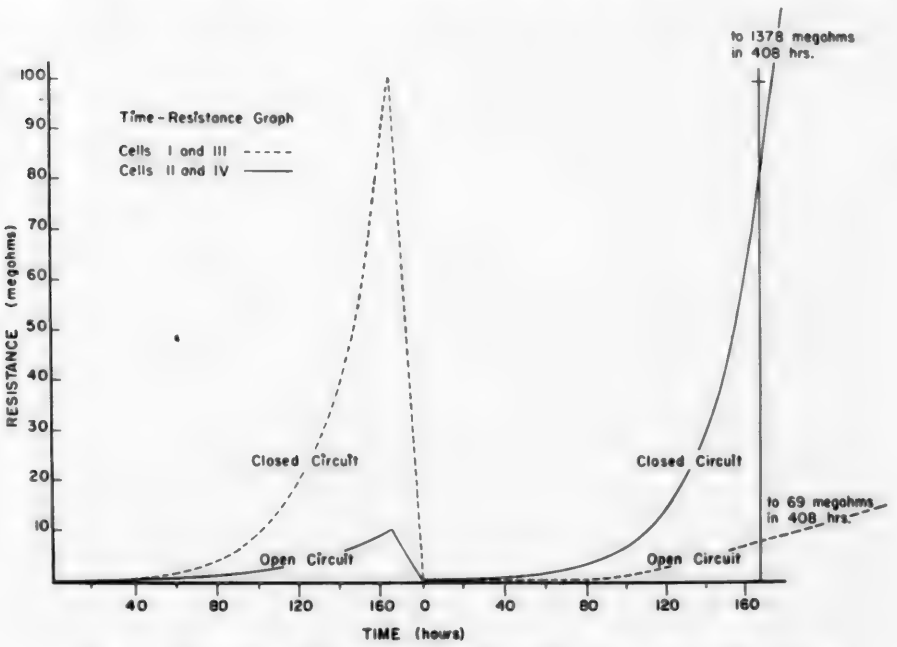

Fig. 4.—Comparison of wet versus dry and closed- versus open-circuit cells.

been on closed circuit now were placed on open circuit, and vice versa. The results are graphically depicted in Fig. 4. The pattern is repetitious. Resistance is low when the margins are exposed but then rises when the cells are resealed. As found from previous tests, the closed-circuit cells have a greater rate of increase than the open-circuit ones. Furthermore, when the cells were interchanged and those on open circuit were placed on closed circuit, the pattern was still the same.

The significance of this chart and these data is first that they are repetitious, thus indicating that the phenomenon is not peculiar to a particular cell. When

the process was reversed, cells which had been on closed circuit and now were placed on open circuit behaved just the same as if the procedure had been reversed. Cells which had been exposed for considerable time (fifty hours) to saline solution recovered their high insulating ability when resealed. Thus these data again illustrate the significant role played by moisture upon the electric resistance of the cement.

TABLE III

TIME-RESISTANCE CHART: AMALGAM, ZINC PHOSPHATE, COPPER CELLS; EXPOSED AND SEALED MARGINS, OPEN AND CLOSED CIRCUITS

CELL NUMBER	RESISTANCE IN MEGOHMS											
	Margin exposed to 1 % NaCl solution for:											
	19 HR.	28 HR.	48 HR.	65 HR.	91 HR.							
I	0.384	0.302	0.335	0.396	0.320							
II	0.354	0.332	0.180	0.195	0.260							
III	0.225	0.350	0.260	0.260	0.085							
IV	0.225	0.394	0.290	0.276	0.165							
	Removed from NaCl solution, resealed I and III on closed circuit, II and IV on open circuit											
	1 HR.	2 HR.	3 HR.	5 HR.	30 HR.	45 HR.	69 HR.	116 HR.	164 HR.			
I	0.130	0.170	0.245	0.195	0.485	1.140	6.06	18.95	100.7			
II			0.444	0.315	0.466	0.450	0.880	35.60	9.60			
III	0.065	0.065	0.040	0.045	0.244	0.450	0.688	60.5	193.0			
IV			0.095	0.110	0.165	0.172	1.38	4.56	10.03			
	Margins exposed to 1% NaCl for 50 hr., resealed I and III on open circuit, II and IV on closed circuit											
	1 HR.	3 HR.	8 HR.	24 HR.	50 HR.	73 HR.	99 HR.	124 HR.	168 HR.	212 HR.	237 HR.	408 H
I	0.500	0.465	0.538	0.522	0.538	0.675	1.80	3.01	9.18	12.77	14.42	69.
II	0.103	0.100	0.053	0.058	0.120	0.138	0.460	1.36	10.9	33.6	44.3	90
III	0.062	0.073	0.082	0.078	0.062	0.148	0.141	4.71	15.1	26.9	32.3	19
IV	0.422	0.387	0.378	0.415	0.860	2.09	7.02	17.3	107.0	128.0	141.0	137

EFFECT OF CONTACT AREA

The effect of the area of the contacting surface on the resistivity of dental cement was also studied. Cells were constructed similar to those used in the other tests with the only variable being the contact area. The thickness of the cement was maintained at 1 mm., but the area was increased from 38.45 sq. mm. to 290 sq. mm. on each surface. The cells were run dry and in the B type of circuit with the electromotive forces bucking. (The B circuit, it will be recalled, is the circuit with an external source of electromotive force and a limiting resistance to control the current to an initial value of approximately 0.5 microampere.) The connections can be reversed by a double-pole, double-throw switch in order to obtain readings necessary for the calculation of cell resistance. These data are shown in Table IV. It is noted that the resistance decreased with these larger cells, and when these values are compared with the normal dry specimens of previous tests, the resistance is only a small percentage of their values. At 1,200 hours most of the smaller cells had reached 1,000 megohms, while the large cells showed an average of only 3.62 megohms. Area does affect conductivity and, as expected, an increase in area reduced the resistance. It is fundamentally true that at low frequency A.C. and D.C., resistance, for example, copper wire, varies inversely as the cross-sectional area. If the cross-

sectional area is doubled, the resistance will become one-half. The decrease here, however, seems to be much greater than a simple inverse ratio of the area increase. Just why this is so remains a problem to be investigated.

TABLE IV

TIME RESISTANCE CHART: AMALGAM, ZINC PHOSPHATE, COPPER CELLS; CIRCUIT B, CELLS ARE SEALED AND ON CLOSED CIRCUIT; INCREASED CONTACT AREA

TIME OF READING AFTER ASSEMBLY (HR.)	CELL NUMBER AND RESISTANCE IN MEGOHMS										
	I	II	III	IV	V	VI	VII	VIII	IX	X	AVG.
2	0.06										0.06
4	0.08	0.17									0.13
8	0.09	0.20	0.20								0.17
16	0.12	0.15	0.12	0.23							0.16
32	0.24	0.04	0.16	0.38	0.26						0.22
64	0.19	0.19	0.33	1.09	1.23	0.44					0.58
128	0.54	0.40	0.42	1.32	1.76	0.35	0.55				0.76
256	1.24	0.38	0.88	1.44	2.46	0.43	0.33	0.91			1.01
384	0.94	0.34	0.50	1.72	2.79	0.34	0.47	1.74	0.87		1.08
512	1.22	1.11	1.33	2.30	4.01	0.52	0.92	1.93	0.73	1.40	1.55
768	1.10	0.94	1.37	1.41	3.80	0.10	0.10	1.58	0.73	1.55	1.56
1,200	1.81	1.74	4.84	3.73	11.30	0.71	1.29	5.30	1.53	3.92	3.62

Possibly this change in resistance with increase of contact area is only of academic interest. The actual area used here is in excess of the cement contact area of the average restoration. Furthermore, if resistance values are in excess of 3 megohms, there will still be adequate insulating ability. The values of generated electromotive force measured by Schriever and Diamond[2] showed a maximum value of approximately 500 millivolts. Neglecting all other resistance in the circuit except the 3 megohms of the cement, the calculated current would be about 0.17 of a microampere. This is obtained merely by employing Ohm's law: voltage divided by resistance equals current. This value of current is about 35 per cent of the values calculated by Schriever as actually flowing in the mouth. It is felt that even with 3 megohms as a minimum value of resistance, the cement is still serving to insulate effectively the circuit. Of course, with higher values, the insulating ability would be increased.

EFFECT OF TEMPERATURES

Tests were made to determine whether temperature influenced the cell resistance. The range of temperatures used varied from 32° to 100° F. The resistance was recorded for the various temperature increments with a series of six cells. It was found that as temperature increased, resistance decreased, and vice versa. It is felt that while this is a variable, the conditions in the mouth, as far as temperature is concerned, are fairly constant. All of the readings in this investigation have been made at room temperature, which did not vary appreciably during the course of the study. While the values can be corrected to mouth temperature, the conclusions drawn will still be the same, and only the actual magnitude will be changed.

For example, a series of readings taken at various room temperatures for a particular cell were:

Time (hr.)	E_s (megohms)	° F.	E_s *converted to 98° F.* (megohms)
0.5	0.245	88	0.178
8	0.180	89	0.126
21	0.120	89	0.084
32	0.170	86	0.129
128	0.110	91	0.073
256	0.060	92	0.038
512	0.030	83	0.025

The conversion, comparing Column 2 and Column 4, shows that the resistance does vary with temperature. While the percentage of change is marked, the alteration in the actual resistance is not significant. The magnitude of resistance shows that the insulating ability is not good for the cement when tested under these conditions, and the same conclusion would be reached whether a temperature of 85° or 98° F. is considered. The conversion does not change a poor insulator into a satisfactory or a good one.

SUMMARY

While previous tests showed zinc phosphate cement to be an effective electric insulator when maintained in a dry condition, this investigation has indicated that its insulating ability is questionable if the cement is exposed to moisture. The resistance pattern of wet cells is entirely different from that of dry cells.

The resistance of the cement was found to be a function of the contact area; the larger the area, the less was the resistance. However, the variation in surface area which occurs in the clinical restoration probably does not significantly alter the insulating characteristics of the cement.

The resistance varied in respect to the temperature at which the cell was maintained. Temperature fluctuation during the investigation was not, however, great enough to alter materially the values observed.

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REACTIONS OF FLUORIDE ION WITH POWDERED ENAMEL AND DENTIN

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A REPORT on reactions of the fluoride ion with synthetic hydroxyapatite was presented recently.[1] It was shown that with relatively pure hydroxyapatite there is a gradual change in the type of reaction as the fluoride concentration increases. With a high ratio of liquid phase to solid (10 L. of solution to 2.5 Gm. of apatite), fluorapatite is formed at the lowest concentrations of F^- and gradually, as the fluoride concentration increases beyond 100 ppm, more and more of the fluoride is converted to calcium fluoride. With a low ratio of liquid to solid (100 ml. of solution and 2.5 Gm. of apatite), the formation of fluorapatite is the chief reaction up to a concentration of 0.2 per cent fluoride; beyond this point, more and more calcium fluoride is formed.

Several mechanisms for the reaction of fluoride with enamel, dentin, and bone have been suggested. Neuman, Neuman, Main, O'Leary, and Smith[2] have shown that fluorapatite is formed below the solubility product of calcium fluoride. Falkenheim and Hodge,[3] and Volker, Hodge, Wilson, and Van Voorhis[4] have shown that surface adsorption of fluoride takes place. The double decomposition of apatite with high levels of fluoride has been demonstrated by Gerould,[5] Scott, Picard, and Wyckoff,[6] and others. This paper presents the results of the extension of the work on synthetic hydroxyapatite to powdered enamel and dentin and an attempt to correlate these findings with those of other workers.

EXPERIMENTAL

Preparation of Enamel and Dentin Samples.—Several thousand human teeth were cleaned of fillings and decayed material with a dental bur, crushed in a steel tube, ground to pass a 60 mesh sieve, and fat extracted with alcohol and ether. Enamel and dentin were then separated by the procedure of Manly and Hodge.[7] Each sample was then separated into twelve sieve fractions, and surface areas from four fractions each of enamel and dentin were obtained. The surface areas and chemical analyses are shown in Table I. For most of the work, a mixed sample made up from a number of these screened samples (excluding –325 fine) was used.

Treatment of Enamel and Dentin With Fluoride.—Samples of both enamel and dentin were treated in two series. In one, 2.5 Gm. of solid was added to 10 L. of solutions ranging from 2 to 2,000 ppm fluoride as NaF. In the other, 2.5 Gm. samples were treated with 200 ml. of solution ranging from 0.05 to 2 per cent fluoride as NaF. These concentrations were used to simulate the

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fluoride concentrations which may occur in natural waters and those used in the topical application of fluoride to the teeth. The first series, made up in 12 L. flasks, and the second, made up in 250 ml. polyethylene bottles, were placed in an incubator room at 37° C. and shaken daily over a period of three to four weeks, then filtered, and dried at 110° C. The filtrates from the concentrated solutions included the washings and were made up to 1 L. One liter of filtrate from each of the dilute solutions was reserved for analysis.

TABLE I

ANALYTICAL DATA ON ENAMEL AND DENTIN SAMPLES

SAMPLE	AREA (M^2 PER GM.)	CaO (%)	P_2O_5 (%)	Ca:P (MOLES)	MgO (%)	CO_2 (%)	Na (%)
Mixed enamel	—	50.89	40.02	1.610	0.53	3.01	0.80
60-80 Mesh	0.94	51.27	40.15	1.616	0.53	2.89	—
100-120 Mesh	1.2	51.13	40.06	1.616	0.47	—	—
200-230 Mesh	1.5	50.90	40.29	1.600	0.39	—	—
-325 (Fine)	9.7	49.11	38.53	1.614	0.57	—	—
Mixed dentin	—	37.29	29.56	1.597	1.31	3.21	0.71
60-70 Mesh	3.2	37.08	29.28	1.603	1.22	3.22	—
100-120 Mesh	3.9	37.17	29.45	1.598	1.21	—	—
200-230 Mesh	4.0	37.48	29.87	1.590	1.30	—	—
-325 (Fine)	11.2	44.82	35.24	1.610	0.84	3.00	—

Electrolytic Conductivity.—The conductivity of a separate series of solutions (0.1 Gm. solid in 250 ml. H_2O) containing 1 to 1,000 ppm of fluoride ion as NaF, mixed with enamel in one series and dentin in another series and equilibrated for one to two weeks at 37° C., was measured in the apparatus previously described.[1]

Analytical.—Samples were analyzed for calcium, phosphorus, and fluoride as described previously,[1] except that a triple precipitation of calcium was employed to ensure a more complete separation of the magnesium. In the solid samples, magnesium was determined in the filtrate of the calcium determination by double precipitation as magnesium ammonium phosphate, as described by Kolthoff and Sandell.[8] Magnesium was determined in the filtered solutions by the thiazole yellow method, as modified by Young and Gill.[10] Carbon dioxide was determined either by evolution with $HClO_4$ and absorption of the dried gas in a weighing bulb, a modification of the standard procedure as given by Kolthoff and Sandell,[8] or by measurement of the volume of evolved gas in a Warburg apparatus. Good agreement was obtained by the two methods. Sodium was determined in the Beckman Model DU Flame Spectrophotometer.[9] Interference of calcium and phosphorus was overcome by adding similar amounts to the sodium standards.

RESULTS

Treatment of Enamel and Dentin With Dilute and Intermediate Fluoride Solutions (Table II).—The methods of calculation previously used[1] with hydroxyapatite are not completely applicable here because of hydrolysis of the samples. The inorganic portion of enamel and dentin consists of hydroxy-

apatite with varying Ca:P ratios caused by the adsorption of acid phosphate ions on crystal surfaces along with other ions including carbonate, citrate, sodium, and magnesium.[11] In dilute solutions, some of these ions are removed from the surfaces with resultant change in the composition of the solid. Thus, as shown in Table II, the Ca:P ratio at 2 ppm fluoride has increased from 1.610 to 1.627 in the enamel and from 1.597 to 1.642 in the dentin. Since at this concentration the increase cannot be due to the formation of insoluble calcium fluoride, it is most probably caused by the removal of some of the adsorbed acid phosphate ions into solution. This is also indicated by the calcium and phosphate content of the filtrates. Also, about 30 per cent of the magnesium has been leached from the dentin (1.3 per cent to 0.9 per cent) and somewhat less from the enamel (see Tables I and II).

TABLE II

TREATMENT OF ENAMEL AND DENTIN WITH DILUTE AND INTERMEDIATE FLUORIDE SOLUTIONS*

SAMPLE	ORIGINAL SOLUTION F (γ/ML.)	SOLID CaO (%)	P_2O_5 (%)	Ca:P MOLES	F (%)	MgO (%)	Na (%)	CO_2 (%)	LIQUID P (μM)	Ca (μM)	Mg. (MG.)
	0	—	—	—	—	—	—	2.78	260	530	
Mixed enamel	2	51.47	40.04	1.627	0.09	0.48	0.71	—	260	350	
(1.610)†	7	51.59	40.07	1.630	0.12	0.42	0.71	2.62	260	290	
	10	51.41	39.90	1.631	0.15	0.46	—	—	260	310	
	25	51.49	40.03	1.628	0.21	0.48	—	—	270	270	
	50	51.85	40.25	1.631	0.28	0.47	—	—	210	210	
	100	51.52	39.85	1.637	0.28	0.48	—	—	370	290	
	500x	51.33	39.57	1.642	0.47	0.46	0.77	—	275	30	
	1,000x	51.31	39.35	1.651	0.77	0.53	—	2.66	530	25	
	2,000x	51.53	38.58	1.691	1.58	0.55	0.90	—	810	60	
	0	—	—	—	—	—	—	3.08	578	770	8.2
Mixed dentin	2	40.16	30.96	1.642	0.56	0.92	0.39	2.75	450	430	7.9
(1.597)†	7	40.04	31.06	1.632	0.86	0.95	0.42	—	450	310	6.8
	10	39.75	30.88	1.630	0.99	0.95	—	—	390	250	6.3
	25	40.08	31.09	1.632	1.25	0.95	—	—	450	290	5.4
	50	40.10	30.87	1.645	1.44	1.07	0.43	—	480	180	4.7
	100	39.92	30.35	1.665	1.55	1.09	0.46	—	520	180	4.6
	500x	40.45	30.39	1.685	1.96	1.29	0.70	—	550	45	0.5
	1,000x	40.15	29.91	1.699	2.22	1.35	0.77	2.51	565	43	—
	2,000x	40.28	29.72	1.716	4.40	1.27	1.93	2.52	970	—	—

*Two and one-half grams of enamel or dentin treated with 10 L. of solution, except those marked "x," which were treated with 5 L.

†Ca:P ratio of original material.

The Ca:P ratios of both the enamel and dentin change but slightly on treatment with solutions containing from 2 ppm through 50 ppm fluoride. The results of analyses also show that the solubility of the solid (as shown by the dissolution of calcium and phosphorus) decreases slightly over this range and considerably between 0 and 2 ppm because of the formation of fluorapatite, which is less soluble than hydroxyapatite.

Another reaction which might take place which would not change the Ca:P ratio is the adsorption of fluoride on the crystal surfaces by exchange with CO_2, probably as bicarbonate ion. This exchange is suggested by the decrease in CO_2 content when fluoride content increases, although the evidence is insufficient for definite conclusions to be drawn. Other negative ions, such

as citrate, may also be exchanged. Another possibility for uptake of fluoride is by direct adsorption. In order to keep the crystals electrically neutral, a positively charged ion must be adsorbed with the negatively charged F^-. In the case of NaF, this cation would be Na^+. Thus a measure of the Na content in the solid should give some idea of the F^- uptake by direct adsorption. These results are also shown in Table II. The adsorption on enamel surfaces is slight over most of the range, but is considerable on the dentin, and a large portion of the F^- is apparently adsorbed directly at 2,000 ppm, as the F^- cannot be accounted for by other mechanisms. The possibility that the organic matrix is involved in the adsorption has not been eliminated. An analysis for sodium was made on hydroxyapatite Sample II treated with fluoride ion over the range 2 to 1,000 ppm as described previously.[1] No adsorption at all took place except at 1,000 ppm, where a very slight amount occurred (0.08 per cent).

In the case of the dentin, and to a limited extent of the enamel, another reaction occurs. Calculations of the solubility product of MgF_2 show that as the fluoride concentration goes up, the solubility product is exceeded, and more and more of the magnesium leached from the solid is reprecipitated as MgF_2 and filtered off with the solid.

At 500 ppm and above, considerable amounts of calcium fluoride are formed by double decomposition. In this range the reactions of enamel and dentin are similar in respect to CaF_2 formation. This is indicated by the considerable increase in the Ca:P ratio and also by the increase in the phosphate content of the filtrates. The additional increase in fluoride content of the dentin over the enamel at 2,000 ppm is due to adsorption, as indicated by the high sodium adsorption.

Treatment of Enamel and Dentin With Concentrated Fluoride Solutions (Table III).—In the interpretation of these figures, as with those in Table II, the methods used in the calculations with synthetic hydroxyapatite[1] are not entirely applicable. Calculations of the Ca:P ratio of the first few enamel samples corrected for the presence of CaF_2, as was done with hydroxyapatite, gives approximately the original ratio of 1.610. This would indicate approximately 100 per cent CaF_2 formation. However, the phosphate released is not sufficient to account for all the fluoride in the solid, particularly at 0.19 per cent F^-. If the phosphate content in moles is multiplied by 3, or even 3.3, to give moles of phosphate equivalent to moles of fluoride (assuming the formation of CaF_2 by double decomposition), only about one-half the fluoride is accounted for. Since the samples subjected to lower fluoride concentrations would be even less likely to form CaF_2 exclusively, it is believed that solution of adsorbed phosphate has occurred to some extent and that a part of the fluoride has formed fluorapatite, part adsorbed on the crystal surfaces directly, and possibly part exchanged with CO_2 on the surfaces. At higher concentrations, particularly at 1.9 and 2.0 per cent, although some F^- adsorption has occurred in the enamel (as indicated by the sodium content), the major portion of the fluoride has formed CaF_2. In the dentin, although CaF_2 has similarly formed, considerable fluoride adsorption has also occurred.

TABLE III

TREATMENT OF ENAMEL AND DENTIN WITH CONCENTRATED FLUORIDE SOLUTIONS*

SAMPLE	ORIGINAL SOLUTION F (%)	SOLID CaO (%)	SOLID P_2O_5 (%)	SOLID Ca:P MOLES	SOLID F (%)	SOLID Na (%)	SOLID CO_2 (%)	LIQUID P (μM)	LIQUID Ca (μM)	LIQUID P (μMx3)	SOLID F (μM)
Mixed enamel (1.610)†	0.048	51.17	39.84	1.626	0.30	0.85	—	103	34	309	394
	0.095	51.05	39.73	1.627	0.30	—	2.75	100	17	300	394
	0.190	51.13	39.76	1.628	0.38	—	—	86	8	258	500
	0.475	51.27	39.55	1.641	0.50	—	—	213	11	639	658
	0.950	—	—	—	—	—	2.58	370	13	1,010	2,660
	1.900	51.01	38.37	1.683	2.04	1.07	—	810	25	2,430	2,680
	2.000x	52.12	38.49	1.714	2.26	—	—	1,162	4	3,486	2,970
	0.000	—	—	—	—	—	3.20	—	—	—	—
Mixed dentin (1.597)†	0.048	39.28	29.99	1.658	1.40	—	2.76	307	—	921	1,840
	0.095	39.87	30.24	1.669	1.56	0.75	—	345	7	1,035	2,050
	0.190	40.25	30.63	1.664	1.60	0.81	—	339	2	1,017	2,100
	0.475	40.32	30.47	1.675	2.00	1.01	—	400	3	1,200	2,630
	0.950	40.31	29.71	1.718	3.12	1.40	2.56	620	3	1,860	4,100
	1.900	41.09	28.55	1.822	4.60	—	2.55	1,357	7	4,071	6,050
	2.000x	41.16	28.91	1.802	4.40	0.96	—	1,065	5	3,195	5,790

*Two and one-half grams of enamel or dentin treated with 200 ml. of solution, except those marked "x," which were treated with 500 ml.
†As in Table II.

Effect of Surface Area (Table IV).—The reactivity of enamel and dentin as indicated by F^- uptake increases with increase in surface area, although not in direct proportion. This is also shown by the increased amount of hydrolysis with decreasing particle size. The most finely divided dentin sample does not follow the expected series. Its analysis, however, seems to resemble enamel more than dentin, and apparently these finest particles failed to separate effectively in the Manly and Hodge procedure. This is also the case with the enamel but to a lesser extent. The dentin particles are much more reactive than the enamel particles, which cannot be accounted for solely on the basis of surface area. That the inner enamel surfaces are less accessible than those of bone has been pointed out previously by Hendricks and Hill.[11] Dentin would be expected to be somewhat similar to bone in this respect.

TABLE IV

EFFECT OF SURFACE AREA ON REACTION WITH FLUORIDE*

SAMPLE	SOLID CaO (%)	SOLID P_2O_5 (%)	SOLID F (%)	SOLID MgO (%)	SOLID Ca:P MOLES	SOLID ORIGINAL Ca:P	LIQUID P (μM)	LIQUID Ca (μM)	LIQUID EXCESS P (μM)	SOLID F (μM)
Enamel:										
0.94 M^2/Gm.	51.36	40.15	0.09	0.50	1.619	1.616	190	230	0	113
1.2 M^2/Gm.	—	40.44	0.10	0.47	—	1.616	230	290	0	125
1.5 M^2/Gm.	51.47	40.20	0.12	—	1.621	1.600	190	250	0	152
9.7 M^2/Gm.	50.43	39.03	0.39	—	1.636	1.614	320	305	0	502
Dentin:										
3.2 M^2/Gm.	39.68	30.80	0.69	1.17	1.628	1.603	360	290	0	907
3.9 M^2/Gm.	39.99	30.88	0.91	1.09	1.639	1.598	450	—	0	1,200
4.0 M^2/Gm.	40.70	31.15	1.07	0.94	1.650	1.590	550	410	0	1,410
11.2 M^2/Gm.	48.14	37.06	0.83	0.63	1.645	1.610	480	420	0	1,090

*Two and one-half grams of sample treated with 10 L. of solution containing 7 ppm fluoride.

Electrolytic Conductivity.—Conductivity measurements indicate, as with synthetic hydroxyapatite,[1] that there is no sudden change in the type of reaction with fluoride as concentration increases. A plot of the results consists of straight lines slightly displaced. The enamel curve is above the synthetic apatite, and the dentin is above the enamel as each releases more ions to the solution by hydrolysis or desorption.

DISCUSSION

The reactions of enamel, and particularly of dentin, have been shown to be much more complicated than those of synthetic hydroxyapatite[1] in the presence of fluoride ions. Thus the hydroxyapatite appears to form fluorapatite at low fluoride concentrations and then gradually more and more calcium fluoride as the concentration increases. Fluoride ion, however, reacts with dentin or ions eluted or dissolved from dentin in several different ways; namely, (a) possible exchange with CO_2 on the crystal surfaces, (b) direct adsorption, (c) precipitation as magnesium fluoride of the magnesium released from crystal surfaces, (d) double decomposition at high fluoride levels to form CaF_2 and Na_2HPO_4, and (e) formation of fluorapatite by exchange with hydroxyl ion. Enamel reacts similarly, but to a much more limited extent.

Assuming that the previously mentioned mechanisms explain the ways in which fluoride ion reacts with powdered enamel and dentin, it is possible, from the results in Table II and Table III, to calculate the approximate amount of fluoride in the solid contributed by each of these reactions.

(a) *Exchange With CO_2.*—Exchange with carbonate may take place to a limited extent as indicated by the decrease in CO_2 content. If it is assumed that the CO_2 is present as bicarbonate, as indicated by Neuman, Neuman, Main, O'Leary, and Smith,[2] each molecule of CO_2 lost from the solid would be equivalent to one of fluoride gained. In the dentin this would amount to about 0.1 per cent F^- at 2 ppm and 0.25 per cent at 2,000 ppm F^-. In the enamel this exchange probably occurs to a maximum of 0.1 per cent F^- at 2,000 ppm.

(b) *Adsorption.*—If sodium adsorption may be used as an indication of fluoride adsorption, a mole for mole calculation may be made. In the dentin this would amount to about 0.01 per cent F at 2 ppm and 1.3 per cent at 2,000 ppm. In the enamel a maximum of about 0.2 per cent is reached at 2,000 ppm.

(c) *Magnesium Fluoride Precipitation.*—The elution in distilled water of 8 mg. of magnesium from the 2.5 Gm. of dentin, amounting to about 25 per cent of the total magnesium content, is additional proof that dentin, as indicated by Hendricks in the case of enamel and bone,[11] varies in composition due to adsorbed ions on the surfaces. The Mg is desorbed in a dilute aqueous solution, and then, as the fluoride content of the solution increases, the solubility product of MgF_2 is exceeded, and thus more and more Mg is precipitated as MgF_2. This is filtered off with the solid and included in the fluoride content of the solid. The difference between the magnesium content of the filtrate from the water-treated dentin sample and a fluoride-treated dentin sample gives the amount of Mg carried down with the solid by

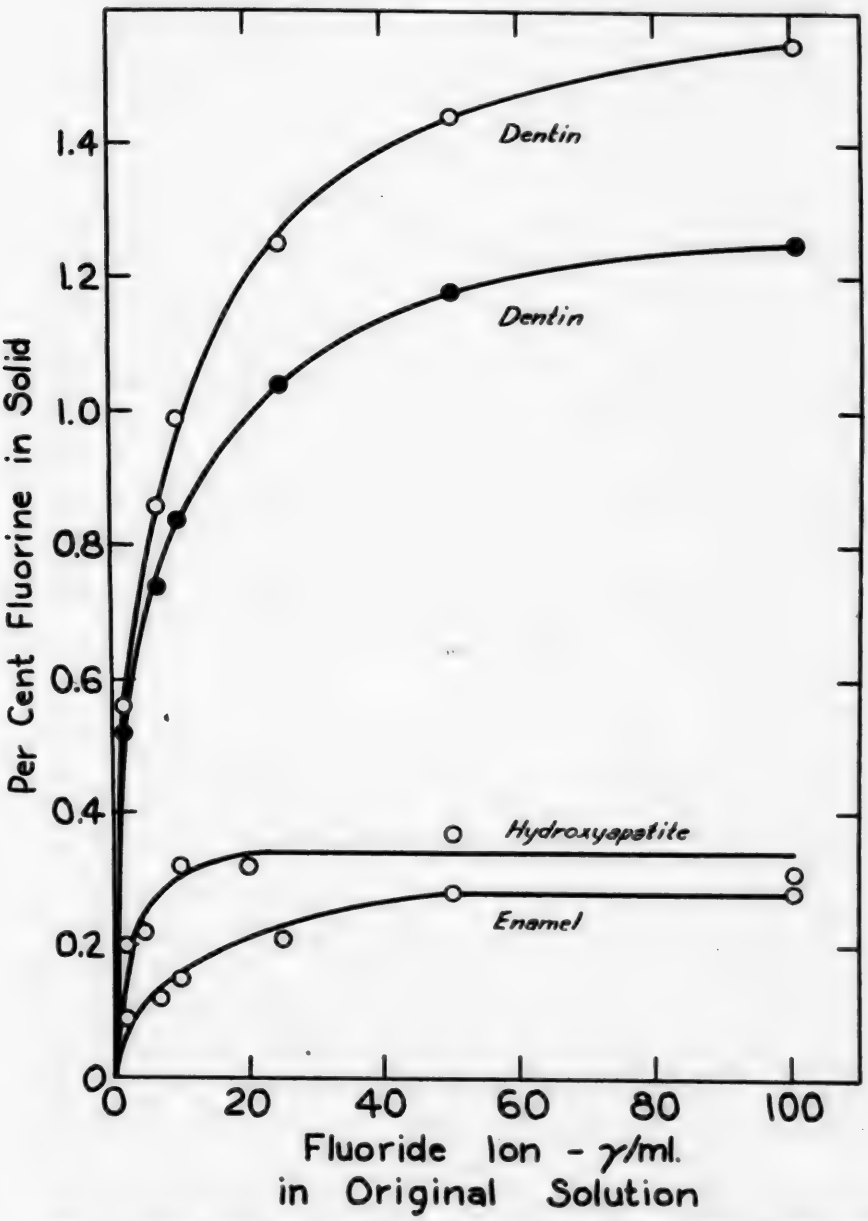

Fig. 1.—Graph showing the leveling off of fluoride uptake as formation of fluorapatite is only or chief product formed up to 100 ppm. The lower dentin curve with the solid black circles has been adjusted for dissolution of Mg from the solid.

fluoride. Assuming this to be MgF_2, the fluoride content may be calculated. In the dentin this amounts to 0.02 per cent F^- at 2 ppm fluoride and 0.5 per cent F^- at 2,000 ppm. In enamel a maximum of 0.1 per cent is reached.

(d) *Calcium Fluoride Precipitation.*—Calcium fluoride is formed by double decomposition of NaF and $Ca_{10}(PO_4)_6(OH)_2$ when the fluoride content of the treating solution becomes sufficiently great. This is indicated by a rise in Ca:P ratio of the solid and also by a rise in the phosphorus content of the filtrate, as shown previously.[1] A 2 ppm treating solution does not furnish sufficient fluoride to exceed the solubility product of CaF_2. In dentin at 2,000 ppm, the F^- contributed by the formation of CaF_2 amounts to about 1.2 per cent, and in enamel to about 1 per cent.

(e) *Fluorapatite.*—The content of fluorapatite is determined by a fluoride balance. The summation of fluoride contributed by all the other mechanisms previously mentioned subtracted from the total fluoride content leaves a balance due to fluorapatite. This amounts to about 0.4 per cent F^- at 2 ppm, and 1.2 per cent F^- at 2,000 ppm in the dentin, and reaches a maximum of about 0.2 per cent in the enamel. In Fig. 1 are shown plots of the concentration of fluoride in the solid versus fluoride ion concentration in the original solutions taken from Table II. The lower dentin curve has been adjusted by subtracting the MgF_2 content of the solid. The leveling off of these curves as they reach 0.2, 0.3, and 1.2 per cent, respectively, is indicative of the formation of fluorapatite almost exclusively up to 100 ppm. Above this fluoride concentration, the curves again rise as CaF_2 is formed by double decomposition.

Reactions of enamel or of exposed dentin in vivo might differ qualitatively from in vitro reactions because of the presence of saliva and other substances. It would not be expected that magnesium would be dissolved from the tooth in most cases. However, the formation of fluorapatite and the direct adsorption of fluoride could take place, as could the double decomposition reaction to form calcium fluoride.

The mechanism of the inhibition of caries by fluorine is still a matter of conjecture. However, it seems reasonable to believe that chemical reactions in teeth exposed to fluorides, such as are found in natural and fluoridated drinking water, result in the formation of fluorapatite. Topically applied fluoride forms calcium fluoride (when a 2 per cent NaF solution is employed), which is probably dissolved in time leaving fluorapatite and adsorbed fluoride. It is also possible that some of the adsorbed fluoride and some of the calcium fluoride is gradually converted to fluorapatite during the course of time.

SUMMARY

It would appear from the results of this study that at least five different reactions may take place between powdered dentin and enamel (or ions dissolved from them) and fluoride ion in vitro. The approximate order of magnitude of these various reactions has been estimated. Additional evidence for the chemical nature of dentin and enamel has been presented. The

reactions which may occur in teeth during the in vivo exposure to fluoride from fluoridated drinking water or by topical application are suggested.

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EFFECT OF pH AND STATE OF OXIDATION OF DIFFERENT FLUORIDES IN THE DRINKING WATER ON DENTAL CARIES AND FLUORINE STORAGE IN THE RAT

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INTRODUCTION

A CONSIDERABLE number of clinical studies[1-5] have attempted to determine the optimal conditions for topical fluoride treatments. In vitro work has been concerned with the possibility of finding a fluoride compound with a greater degree of protectiveness than sodium fluoride.[6-12] It has been considered that the beneficial action of topically applied fluoride may be enhanced by decreasing the pH of the solution,[12-15] but a clinical study using an acidulated sodium fluoride solution on adults was negative.[16] An acidulated sodium fluoride mouthwash appeared to increase caries in a clinical study.[17] However, stannous fluoride has been shown to be more effective than sodium fluoride in reducing the initiation as well as the severity of dental caries in children[18] when applied at one-half the fluoride concentration recommended for sodium fluoride and at pH 2.9. Therefore, further work appears necessary to clarify the relationship of pH of the fluoride solutions to their effectiveness in reducing dental caries.

EXPERIMENTAL

A total of 273 weanling rats* were divided as to sex and initial body weight into seven different experimental groups. All the rats were given a cariogenic corn diet[10] and drinking water ad libitum. Four groups, each composed of males and females, were given fluoride supplements in the drinking water at two different pH levels, while a fifth group served as a control and received the same water without the addition of any fluoride. The pH of the fluoridized drinking water used was 3.5 or 5.5. The pH of the solutions was adjusted by the addition of either sodium hydroxide or hydrochloric acid. Stannous fluoride, stannous chlorofluoride, and sodium fluoride were used. The fluoride solutions were initially made substantially free of oxygen to minimize the oxidation of the stannous tin in the stannous compounds, since oxidation and hydrolysis have been previously shown to decrease the cariostatic activity of tin fluoride.[12] In addition, all the solutions were prepared fresh each day in a further attempt to decrease the effect of aging of the tin solution.[12]

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*Sprague-Dawley strain, furnished by Carl Wilson, Beech Grove, Indiana.

The method of preparing the oxygen-free fluoride solutions was as follows: A large quantity of fluorine-free distilled water was placed in a Pyrex flask and boiled for one-half hour. Then, a stopper was inserted through which there were two tubes. One was attached to a source of nitrogen which was constantly bubbled through the water until it had cooled to room temperature. The other tube served as an exit for the nitrogen. Portions of this water were then carefully removed and used to prepare fresh solutions each morning, and any remaining in the drinking containers was discarded before the fresh solution was added. Although the water became exposed to air immediately after the drinking containers were filled, these precautions assured the availability of stannous tin for a longer period than would have been possible if no precautions had been taken.

All the animals were started on the experiment when 30 days old and killed by ether inhalation when 170 days old. The heads were removed and stored in 10 per cent formalin until ready for examination, and six femurs from each sex and each group were removed for fluorine determination. The method used for examining the teeth and analysis for fluorine has been reported.[12]

RESULTS AND DISCUSSION

The effects on the incidence of dental caries in the molar teeth are shown in Table I. In both sexes, the stannous chlorofluoride and stannous fluoride at pH 3.5 were superior to sodium fluoride both in their effect on the reduction of caries initiation and caries extension. However, when the pH of the drinking water was increased to 5.5, the effectiveness of the two tin compounds was decreased, substantially, to the level of sodium fluoride at pH 5.5. Hence, these data indicate that at a low pH, tin enhances the effectiveness of fluorine, the effect being more pronounced when the tin and fluorine are provided in the form of stannous fluoride or stannous chlorofluoride.[13] The reason for this cannot be explained at present.

TABLE I

THE EFFECT OF DIFFERENT FLUORIDES AND pH ON DENTAL CARIES IN THE RAT (FLUORINE CONCENTRATION WAS 20 μG PER MILLILITER IN ALL INSTANCES AND THE WATER USED WAS OXYGEN-FREE WHEN IT WAS GIVEN TO THE RATS)

SUPPLEMENT	NUMBER OF ANIMALS	NUMBER OF CARIOUS LESIONS PER RAT	EXTENT	REDUCTION (%)	ASHED FEMUR		
					WEIGHT (GM.)	F CONCENTRATION (μG/GM.)	TOTAL F (MG.)
SnF_2, pH 3.5	41	2.5	0.40	69	0.2195	2,173	0.476
SnF_2, pH 5.5	36	5.4	1.02	31	0.2307	2,165	0.496
SnClF, pH 3.5	40	2.7	0.37	66	0.2331	2,330	0.549
SnClF, pH 5.5	38	5.2	1.04	35	0.2275	2,075	0.470
NaF, pH 3.5	41	3.3	0.46	58	0.2594	3,040	0.779
NaF, pH 5.5	40	4.6	0.88	41	0.2227	2,826	0.639
None	37	7.9	1.79	—	0.2695	300	0.080

These findings at the higher pH differ from others[10, 12] wherein stannous fluoride was more effective than sodium fluoride. However, in previous work

all of the fluoride solutions were either aged preparations[10, 11] or prepared fresh daily,[12] but in no case were such extreme measures taken to reduce oxidation of the tin as in this experiment. Therefore, it seemed important to test under identical conditions the same three fluorides used in this experiment that were prepared fresh each day from oxygen-free water under the same conditions, but not having the pH of the fluoridized water adjusted or the water boiled or kept under nitrogen before making the fluoride solutions. For this experiment, 169 weanling rats were divided as to sex and initial weight and placed into four groups. Three groups received their respective fluorides in the drinking water at the same fluorine concentration used in the first experiment reported in this paper, while the fourth received water without added fluoride and served as a control. The pH of the tin fluorides was about 3.5, while the sodium fluoride was about 5.8. The dental caries data are given in Table II, and demonstrate, as observed in previous experiments,[10-13] that stannous fluoride is decidedly superior to sodium fluoride. Stannous chlorofluoride was equally as effective as stannous fluoride. Thus, it appears that the superiority of stannous fluoride may be related to the pH. However, this cannot entirely explain its more beneficial effect, for stannous fluoride and stannous chlorofluoride are more effective than sodium fluoride at the same lower pH (Table I). Also, it is noted from these two experiments that both the tin compounds were decidedly more effective when prepared in oxygen-free water. For example, stannous fluoride at pH 3.5 prepared in oxygen-free water decreased caries by approximately 70 per cent, while in another experiment the same fluoride under seemingly identical conditions, but prepared in oxygen-containing water, reduced caries by only approximately 50 per cent.

TABLE II

A COMPARISON OF STANNOUS FLUORIDE, STANNOUS CHLOROFLUORIDE, AND SODIUM FLUORIDE AT 20 μG FLUORINE PER MILLILITER IN OXYGEN-CONTAINING DRINKING WATER ON CARIES REDUCTION IN THE RAT

SUPPLEMENT	NUMBER OF ANIMALS	NUMBER OF LESIONS	REDUCTION (%)	FEMUR ASH		
				WEIGHT (GM.)	F CONCENTRATION (μG/GM.)	TOTAL F (MG.)
SnF_2	41	4.3	48	0.2458	2,532	0.623
SnClF	46	4.5	45	0.2767	1,946	0.537
NaF	41	6.2	25	0.2547	3,099	0.800
None	41	8.2	—	0.2608	388	0.101

In the second experiment the sodium fluoride also was less effective than in the first. This would seem to indicate that oxygen-free water even increases the protection of sodium fluoride against dental caries. However, in the second experiment the pH of the sodium fluoride was higher, and it was not controlled. Hence the apparent difference in protectiveness may have been due to a combination of differences in pH and the inherent experimental error.

Also, the storage of fluorine in the femurs indicates differences between the two compounds that cannot be explained on a difference in pH. Tables I and II indicate the storage of fluorine in the skeleton after the animals used in this study received fluoridized drinking water solutions ad libitum for 140 days. Two important facts are evident. First, the two tin fluorides cause the storage of decidedly less fluorine than sodium fluoride and, second, the amount of fluorine stored does not seem to be related to the pH of the fluoride solution. The storage of fluorine from stannous fluoride does not appear to be affected by pH changes, yet its effectiveness in reducing dental caries is 50 per cent less when the pH of the fluoride solution is increased from 3.5 to 5.5. The data also indicate that sodium fluoride causes the storage of more of the element than either of the two tin fluorides. These findings confirm and extend those previously reported,[12, 13] and further indicate the lack of a definite relationship between fluorine storage and the incidence of dental caries in the rat.

SUMMARY

When stannous fluoride and stannous chlorofluoride were given to rats fresh each day in oxygen-free drinking water at a fluorine concentration of 20 μg per milliliter and at pH 3.5, they were decidedly more effective in reducing the initiation and extension of dental caries than was sodium fluoride at the same pH. However, the marked effectiveness of these two tin fluorides is decreased when the pH is increased to 5.5, while sodium fluoride is not affected to the same degree.

The degree of dental caries resistance does not seem to be related to the storage of fluorine in the femur, and the pH of the fluoride solution apparently is not related to its storage.

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THE EFFECT OF TOPICAL FLUORIDE ON DENTAL CARIES EXPERIENCE IN ADULT FEMALES OF A MILITARY POPULATION

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INTRODUCTION

SIGNIFICANT reductions in caries activity have been reported by several investigators following the topical application of fluoride.[1-3] Positive results were obtained in studies on children when treated mouth quadrants were compared with untreated quadrants in the same individuals.

Arnold, Dean, and Singleton[4] observed no reduction in caries activity when whole mouth treatments were applied to adults in a military population. This has been attributed to the fact that the latter investigators used an acidulated fluoride solution and that the applications were made at one sitting. Rickles and Becks[3] failed to observe a reduction in caries activity when mouth quadrants of an adult population were treated with acidulated solutions, whereas positive results were obtained in similar age groups when a neutral solution was used. Klinkenberg and Bibby[1] showed positive results in young adults with neutral fluoride solutions. These investigators emphasized the fact that their experimental group was small, the neutral fluoride having been tried in twenty-five individuals and the acidulated in twenty-two. Recently Kutler and Ireland[5] reported no significant reductions in the dental caries experience of 147 adults when quadrants were treated with a neutral 2 per cent sodium fluoride solution.

The study to be reported is concerned with a female military population (WAVES) who received full mouth treatments with a neutral 2 per cent sodium fluoride solution.

METHODS AND MATERIALS

Two hundred seventy enlisted servicewomen (WAVES), at the Great Lakes Naval Training Center, volunteered for this study in the fall and winter of 1951-1952. Their ages ranged from 19 to 39 years, with an average age of 22 years. Their place of birth and residence for the first eight years of their lives was distributed among thirty-two states, two possessions, and the District of Columbia. Of the total initially examined, 148 were available for a final examination in the winter of 1952-1953. The number of persons initially examined for this study was reduced by their transfer to other duty stations or by release to inactive status. Sixty women in this group had received fluoride treatments and eighty-eight were treated with a placebo solution. More than one-half of these individuals in the experimental group received four successive treatments, while the remaining received three or less, and only five received one treatment.

The opinions expressed in this article are those of the authors and are not to be construed as reflecting the views of the Navy Department or the Naval Service at large.

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Carious teeth, filled surfaces, and missing teeth were noted clinically with a mouth mirror and explorer. This information was recorded by a modified coded system supplied by the United States Public Health Service. The outline permitted scoring of various surfaces of each tooth, anomalies, fluorosis, and, to a limited degree, other defects.

Full month roentgenograms, plus four bite-wing films, were taken on each individual. The gingivae were charted after the method of Massler, Schour, and Chopra.[6] This latter material will be the subject of additional reports. Pre- and postclinical examinations and interpretations of the roentgenograms were made by the same person. Cross-checking was conducted by an investigator apart from this study.

Each individual submitted at least two fasting saliva samples for bacteriologic examination before and after the period of this study. The laboratory procedures included plating the saliva on tomato juice agar as described by Jay and Arnold.[7]

The test group was treated with a neutral solution of 2 per cent sodium fluoride and the control group with a placebo containing 0.9 per cent sodium chloride. Treatment schedules were made at random on alternate individuals.

The clinical technic employed for the application of the solutions was as follows: (1) A thorough prophylaxis was given followed by a water rinse. (2) The teeth were isolated with cotton rolls and a saliva ejector was used. (3) The previously dried teeth were sprayed with the test solution. (4) The teeth were kept moist for the next five minutes by repeated swabbing of all tooth surfaces, with the particular solution being tested. (5) The teeth were then allowed to air dry for four minutes, after which the mouth was rinsed with water. Subsequent semiweekly applications were not preceded by a prophylaxis.

RESULTS

There was a range of eight to fourteen months' lapse between the initial and final examinations. The majority of persons included in this study were in the 21-year-old age group; their duty and environment were similar except for periods of leave.

There was no significant variation in average number of newly decayed teeth in persons receiving one, two, three, and four treatments of topical sodium fluoride.

The bacteriologic studies showed that high salivary lactobacillus counts were associated with high caries activity. It was evident that exceptions prevailed in both instances, a point not warranting discussion here. The influence of the fluoride ion on the lactobacillus counts was not demonstrable when samples were analyzed on termination of this study. Similar fluctuations in the average bacterial counts were observed in the control and experimental groups.*

New carious teeth were tabulated and the resultant figures subjected to statistical analysis (Tables I and II). Carious tooth surfaces were also noted but were not used in this paper.[9] The per cent of WAVES developing new carious teeth during the study was 60 per cent in the group receiving sodium fluoride, as compared to 68 per cent in the control group.

*Subject of future publication.

TABLE I

DISTRIBUTION OF NEWLY DECAYED TEETH AMONG SIXTY WAVES RECEIVING SODIUM FLUORIDE TREATMENT AND EIGHTY-EIGHT RECEIVING PLACEBO (SODIUM CHLORIDE)

NO. NEWLY DECAYED TEETH PER PERSON	SODIUM FLUORIDE NUMBER OF WAVES	PLACEBO NUMBER OF WAVES
0	24	28
1	23	36
2	7	17
3	5	5
4	0	0
5	1	2
Total	60	88

Mean newly decayed teeth per person	0.950	1.079
Standard deviation	1.064	1.046
Standard deviation of mean	0.141	0.112
Standard error of the difference in means	$\sqrt{(0.14)^2 + (0.11)^2} = 0.178$	

$\frac{\text{Dev.}}{\text{Sigma}} = 0.724 = 1.079$

Probability of obtaining an equal or larger deviation = 48 per cent; this is not statistically significant.

TABLE II

THE TOTAL NUMBER OF INITIALLY CARIES-FREE TEETH AND NEWLY DECAYED TEETH IN SIXTY WAVES RECEIVING SODIUM FLUORIDE AND EIGHTY-EIGHT RECEIVING A PLACEBO (SODIUM CHLORIDE)

	CARIES-FREE TEETH	NEWLY DECAYED TEETH	PER CENT NEWLY DECAYED TEETH
Sodium fluoride	1,165	57	4.89
Placebo group	1,765	95	5.38
Total	2,930	152	5.19

5.38 per cent − 4.89 per cent = 0.49 per cent difference.
0.49 per cent ÷ 5.38 per cent × 100 = Per cent reduction of 9.1

The effect of sodium fluoride on the inhibition of newly decayed teeth per person is presented in Table I. The average number of newly decayed teeth per person was 0.95 for the fluoride subjects and 1.08 for those receiving the placebo. The comparison of the difference between these two means is not considered statistically significant $\left(\frac{\text{Dev.}}{\text{Sigma}} = 0.724\right)$. The per cent reduction of the average number of newly decayed teeth in the sodium fluoride over the placebo group is 12 per cent $\left(\frac{1.079 - 0.95}{1.079} \times 100\right)$. Calculation of this difference in terms of percentage reduction is 9.1 per cent (Table II). Since Table I reveals no significance, this percentage reduction is considered to be of no consequence.

SUMMARY

Sixty Navy enlisted women were treated topically with 2 per cent neutral sodium fluoride, and eighty-eight controls with sodium chloride. Two tables are presented to show the results of this form of treatment. There was no significant reduction in caries incidence of persons receiving a sodium fluoride solution over those receiving a placebo. The salivary lactobacillus counts were not affected by this form of treatment.

Acknowledgment.—We wish to thank Dr. Neal Chilton for his review and suggestions of the statistical analysis of this paper.

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THE EVANSTON DENTAL CARIES STUDY

XI. The Caries Experience Rates of 12-, 13-, and 14-Year-Old Children After Exposure to Fluoridated Water for Fifty-nine to Seventy Months

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THE dental caries experience rates of Evanston school children, aged 12, 13, and 14 years, indicated a reduction of 12.19 per cent after these children had been exposed to artificially fluoridated water for a period of twenty-three to thirty-four months.[1]

This report on the dental caries experience rates is for children of the same age, presumably influenced by the same conditions, but with exposure time to fluoridated water increased from twenty-three to thirty-four months to fifty-nine to seventy months at the time of dental examination.

METHOD

The mechanism of the study, with the exception of the material pertaining to rates per hundred teeth and rates per hundred surfaces, has been covered by Blayney and Tucker[2] in their report on the purpose of the study. Measuring caries experience solely on the basis of decayed, missing, and filled teeth per hundred children was thought to be somewhat questionable as that method allows a single tooth to have multiple carious lesions or fillings and still be tabulated as only one decayed, missing, or filled tooth. Bruckner, Hill, and Wollpert[3] conducted a study with a secondary objective of comparing several methods of caries measurement. They concluded that the use of the ratios decayed, missing, and filled teeth to total teeth allowed multiple caries attacks on individual teeth to be partially obscured, although this was not true when the ratios of decayed surfaces to total surfaces were employed.

The expression of dental caries experience rates per hundred tooth surfaces appears to be somewhat more reliable, so in this report rates are expressed in terms of per hundred children, per hundred teeth, and per hundred surfaces. In calculating rates per hundred teeth, teeth present plus teeth extracted due to caries comprised total teeth. Total surfaces were total teeth multiplied by five, as all teeth were considered on the basis of five surfaces. A record of teeth present and teeth missing was made for each child in the study. Only teeth lost due to caries were recorded as missing (extracted). Only teeth which were 50 per cent or more erupted were considered. A carious or filled tooth was, of course, considered regardless of its stage of eruption. A proximal occlusal restoration was considered as two surfaces. Proximal surface lesions or proximal surface fillings were recorded as one surface.

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We have made no attempt to establish a value of missing teeth in terms of surfaces involved with caries. Bruckner, Hill, and Wollpert,[3] in their study of children in the age range of 6 to 16 years, determined a value of 2.7 for 122 posterior, and 1.5 for six anterior, teeth needing extraction. However, at the end of their study the average number of carious and filled surfaces on sixty-eight posterior teeth actually extracted during the year was 2.3. Boyd and Cheyne,[4] in studying the incidence of caries among institutionalized children 6 to 19 years of age, accepted an average of three affected surfaces for each extracted tooth. In this report a loss of five surfaces, whether the tooth was anterior or posterior, was recorded for each extracted tooth.

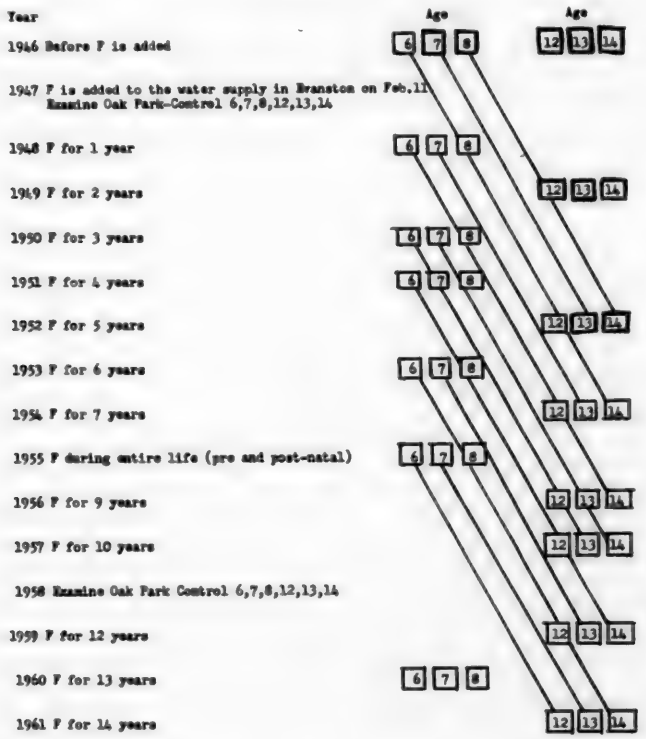
Year
Age
Age
1946 Before F is added
6 7 8
12 13 14
1947 F is added to the water supply in Evanston on Feb.11
Examine Oak Park-Control 6,7,8,12,13,14
1948 F for 1 year
1949 F for 2 years
1950 F for 3 years
1951 F for 4 years
1952 F for 5 years
1953 F for 6 years
1954 F for 7 years
1955 F during entire life (pre and post-natal)
1956 F for 9 years
1957 F for 10 years
1958 Examine Oak Park Control 6,7,8,12,13,14
1959 F for 12 years
1960 F for 13 years
1961 F for 14 years

Fig. 1.—Schema for study.

FINDINGS

Referring to Fig. 1, the schema of the study, the parallel lines running from the 6-, 7-, and 8-year-old children of the 1946 baseline group to the 12-, 13-, and 14-year-old children of 1952 indicate the relationship of the two groups of children. Theoretically the 1946 group is now the 1952 group. However, it was found that 66.80 per cent of the 1,991[5] 6-, 7-, and 8-year-old children examined in 1946 appeared as the 12-, 13-, and 14-year-old children in 1952. Some of the 6-year-old children apparently were lost to the study as

8-year-olds in 1948 when a temporary and unavoidable change was made in the order of school examinations because of a conflict with hearing tests which were being conducted in some schools. The 8-year-old children of 1948 became the 1952 12-year-old group, and this rearranging of the schedule may have been influential in causing some of the students examined in 1946 to be missed in 1948 and again in 1952. Of course there are always a few students ill at the time of examination, some away on vacation, and some families move from one section of the city to another causing a child to transfer from school to school. At the present time we have no way of following such transfers except as we find them in the school which is currently being examined. A few of the students surveyed in 1946 were either a month or two under the age limit for the 6-year-old group or over the limit for the 8-year-old children. These out-of-the-age-range children would not fall into the proper age brackets in 1952 and therefore are not included in any of the tabulations of this study. Of the 1,554 participants in 1952, 1,330 or 85.58 per cent were from the original 1946 baseline group and 14.42 per cent joined the study after 1946. The data given in this report are limited to those children whose entire lives have been on Lake Michigan water.

Differences in Dental Caries Experience Rates, 1946-1952, DMF Permanent Teeth.—The reduction in the decayed, missing, and filled teeth per hundred children is approximately 21.0 per cent for the 12- and 13-year-old group and 12.5 per cent for the 14-year-old children when compared to the same age groups of the prefluoride base line (Table I). When the 1952 figures, after five years of fluoridation, are compared with the results obtained for this age group in 1949, after two years of fluoridation, we see that the DMF rates in 1952 again have been reduced almost twice as much as in 1949.

TABLE I

DECAYED, MISSING, AND FILLED PERMANENT TEETH: RATE PER HUNDRED CHILDREN

YEAR	NUMBER OF EXAMINATIONS			TIME OF EXPOSURE TO 1 PPM F	AGE (YR.)		
	12	13	14		12	13	14
1946	418	688	595	None	763.16	1,009.59	1,165.88
1949	522	677	570	23-34 mo.	681.99	857.02	1,086.84
1952	512	533	509	59-70 mo.	596.87	795.49	1,019.84
Percent differences 1946-1952					R 21.79%	R 21.21%	R 12.53%
I increase R reduction							
Probability of difference due to chance*					0.000	0.000	0.000

*Any probability less than 0.05 is conventionally considered significant. Tests for measuring the significance of the differences between two means were made on these data.

In comparing 1946 and 1947 baseline studies in Evanston and in Oak Park (the control), there was apparently a considerable difference in the rates. When the figures were analyzed it was found that comparisons were being made of dissimilar groups rather than similar or like groups. In the control village of Oak Park, only public school children were studied, while the Evanston group was made up of not only public school children, but children from parochial schools and from one school with only Negro students. When like groups were compared to like groups, the rates were comparable.[1] Since

there was a difference in rates in 1949, it was decided to determine the rates of the same groups after fifty-nine to seventy months of exposure to fluoridated water to compare the changes in the various groups. Table II shows that the public school white children had a percentage reduction in dental caries rates less than the reduction found in both the Foster School students (Negro children only) and the parochial school children. The Foster School children had the lowest DMF rates in 1946, for all ages, (12, 13, and 14), when compared to the rates of the public school white children and the parochial school children. The Foster School group had the lowest rate again in 1952 when compared with the other two groups. The Foster School children also had the greatest percentage reduction of the three groups, with the exception of the parochial school 12-year-old children, whose reduction rates was approximately 1.59 per cent higher. These results give rise to the question: Are we to expect the greatest percentage reduction in the caries rate in those groups having the lowest or the highest DMF rates? In our opinion, more evidence is required before this question can be answered. The fact that there were so few examinations made on the Foster School children may influence these data. It is possible that some additional factors such as hygiene or dietary control may be active in causing this variation in percentage reduction in the DMF rates.

TABLE II

DECAYED, MISSING, AND FILLED PERMANENT TEETH: RATE PER HUNDRED CHILDREN

AGE	PUBLIC SCHOOL (WHITE CHILDREN) NO. EXAMINATIONS: 1,226	PER CENT DIFFERENCES	FOSTER SCHOOL (NEGRO ONLY) NO. EXAMINATIONS: 96	PER CENT DIFFERENCES	PAROCHIAL SCHOOL CHILDREN NO. EXAMINATIONS: 379	PER CENT DIFFERENCES
			Evanston, 1946			
12	707.51		658.82		981.32	
13	946.17		861.76		1,218.13	
14	1,133.33		1,035.71		1,322.22	
			Evanston, 1952			
	No. Examinations: 1041		No. Examinations: 88		No. Examinations: 425	
12	569.45	19.51%	476.00	27.75%	693.38	29.34%
13	746.88	21.06%	600.00	30.38%	918.02	24.64%
14	1,015.27	10.42%	728.57	29.66%	1,077.45	18.51%

Occlusal Surfaces With Pit and Fissure Caries or Fillings.—The reduction in occlusal surface pit and fissure caries or fillings is approximately equal for the 12- and 13-year-old children, with the 14-year-old students showing the smallest percentage difference (Table III); the 12-year-old children's percentage difference was almost twice the percentage reduction found in 1949[1] However, the 13-year-old children showed a reduction in 1952 of 18.42 per cent which was a very slight increase over the 16.6 per cent reduction found in 1949. The 14-year-old children had a 1952 reduction of 11.95 per cent, which was less than the reduction of 13.74 per cent found in 1949. The 1952 percentage reductions were all considered significant.

TABLE III

OCCLUSAL SURFACE PIT AND FISSURE CARIES OR FILLINGS, PERMANENT MOLAR TEETH: RATE PER HUNDRED CHILDREN

YEAR	NUMBER OF EXAMS 12	13	14	TIME OF EXPOSURE TO 1 PPM F	AGES 12	13	14
1946	418	688	595	None	351.67	438.08	471.26
1949	522	677	570	23-34 mo.	313.41	364.99	406.49
1952	512	533	509	59-70 mo.	286.52	357.41	414.93
Per cent differences 1946-1952 I increase R reduction					R 18.53%	R 18.42%	R 11.95%
Probability of difference due to chance*					0.000	0.000	0.000

*Any probability less than 0.05 is conventionally considered significant.

Occlusal Surfaces Free From Pit and Fissure Caries or Fillings.—There was a considerable increase found in the number of occlusal surfaces free from pit and fissure caries or fillings in all the age groups (Table IV). There was a percentage increase in 1952 for the 12-year-old children of more than three times the percentage increase found in 1949.[1] The 13- and 14-year-old children had percentage increases more than twice those found in 1949. While the number of molars with occlusal surface caries experience for the three ages indicated a reduction of only 18.53 per cent, 18.42 per cent, and 11.95 per cent for the 12-, 13-, and 14-year-old groups, respectively, a comparable percentage increase may be expected when considering the molar occlusal surfaces free of caries in the same children. However, this was not so. There was a far greater increase in the rates concerning molars free from occlusal caries or fillings than a decrease in the number with occlusal surface caries or fillings. This unbalanced condition will probably prevail until the rates are considered from the number of permanent molars present, the number with occlusal surface caries experience, and the number free from occlusal surface caries experience rather than as per hundred children.

TABLE IV

OCCLUSAL SURFACES FREE FROM PIT AND FISSURE CARIES OR FILLINGS, PERMANENT MOLAR TEETH: RATE PER HUNDRED CHILDREN

YEAR	NUMBER OF EXAMS 12	13	14	TIME OF EXPOSURE TO 1 PPM F	AGES 12	13	14
1946	418	688	595	None	194.02	190.84	183.02
1949	522	677	570	23-34 mo.	221.46	253.91	238.25
1952	512	533	509	59-70 mo.	290.42	323.26	321.02
Per cent differences 1946-1952 I increase R reduction					I 49.69%	I 69.39%	I 75.40%
Probability of difference due to chance*					0.000	0.000	0.000

*Any probability less than 0.05 is conventionally considered significant.

Maxillary Anterior Teeth (Canine to Canine).—The reductions found in the 1952 examinations were approximately equal for the 12- and 13-year-old children, 24.34 per cent and 23.05 per cent, respectively. The reduction for the 14-year-old children was only 9.06 per cent (Table V). The 12- and 13-year-old group percentage reduction rates in 1952 were approximately twice

the percentage reduction rates found in 1949 but the 14-year-old group in 1952 demonstrated slightly less reduction than that of 1949. It should be stated that the 1952 reduction for the 14-year-old children is not considered significant.

TABLE V

DECAYED AND FILLED MAXILLARY ANTERIOR PERMANENT TEETH, CUSPID TO CUSPID: RATE PER HUNDRED CHILDREN

YEAR	NUMBER OF EXAMS 12	13	14	TIME OF EXPOSURE TO 1 PPM F	AGES 12	13	14
1946	418	688	595	None	127.27	157.26	172.60
1949	522	677	570	23-34 mo.	110.34	135.60	153.56
1952	512	533	509	59 70 mo.	96.29	121.01	156.97
Per cent differences 1946-1952 I increase R reduction					R 24.34%	R 23.05%	R 9.06%
Probability of difference due to chance*					0.002	0.000	0.159

*Any probability less than 0.05 is conventionally considered significant.

Mandibular Anterior Teeth (Canine to Canine).—Although the percentage reductions found in 1952 were greater than those in 1949, except for the 13-year-old group, none of the 1952 reduction rates proved to be significant (Table VI). Apparently, there were too few carious or filled teeth. Again the use of rates per hundred teeth or surfaces probably would express more adequately any change in rate in caries attacks in these teeth.

TABLE VI

DECAYED AND FILLED MANDIBULAR ANTERIOR PERMANENT TEETH, CUSPID TO CUSPID: RATE PER HUNDRED CHILDREN

YEAR	NUMBER OF EXAMS 12	13	14	TIME OF EXPOSURE TO 1 PPM F	AGES 12	13	14
1946	418	688	595	None	31.82	45.20	45.71
1949	522	677	570	23-34 mo.	28.16	35.75	38.25
1952	512	533	509	59-70 mo.	25.00	38.65	36.94
Per cent differences 1946-1952 I increase R reduction					R 21.43%	R 14.49%	R 19.19%
Probability of difference due to chance*					0.246	0.317	0.180

*Any probability less than 0.05 is conventionally considered significant.

Permanent Tooth Surfaces With Pre-Carious Lesions.—The number of pre-carious lesions found in 1952 is less than the numbers found in 1946 and 1949, except for the 12-year-old group, whose pre-carious lesions increased over the 1949 level. The reduction is considered to be significant when compared with the 1946 rates. The increase in the 12-year-old children's pre-carious lesions in 1952 is considered to be significant when compared with the 1949 rate for this age group (Table VII).

Immune Permanent Dentitions.—The 12-year-old children had an increase of 89.59 per cent in the immunity rates when compared to 1946, although this increase was not considered significant. The 13-year-old children did have a considerable percentage increase when comparing the 1952 rate to the 1946

rate. This increase was considered to be significant. As compared to the rate in 1949, the rate of the 13-year-old children in 1952 increased slightly. The 14-year-old children had a rate of less than 1 immune case per hundred children in 1952, and here a reduction of 61.39 per cent was found but was not considered significant (Table VIII). There were so few immune cases and the number per hundred children was so low that the differences when converted to percentages appeared large.

TABLE VII

PERMANENT TOOTH SURFACES WITH PRE-CARIOUS LESIONS: RATE PER HUNDRED CHILDREN

YEAR	NUMBER OF EXAMS 12	13	14	TIME OF EXPOSURE TO 1 PPM F	AGES 12	13	14
1946	418	688	595	None	130.62	139.10	139.33
1949	522	677	570	23-34 mo.	57.85	85.52	92.81
1952	512	533	509	59-70 mo.	81.84†	68.29	91.75
Per cent differences 1946-1952					R 37.35%	R 50.91%	R 34.15%
I increase R reduction							
Probability of difference due to chance*					0.000	0.000	0.000

*Any probability less than 0.05 is conventionally considered significant.
†Increase from 1949 is significant (0.001).

TABLE VIII

IMMUNE PERMANENT DENTITIONS: RATE PER HUNDRED CHILDREN

YEAR	NUMBER OF EXAMS 12	13	14	TIME OF EXPOSURE TO 1 PPM F	AGES 12	13	14
1946	418	688	595	None	1.44	1.02	1.01
1949	522	677	570	23-34 mo.	1.53	2.51	1.75
1952	512	533	509	59-70 mo.	2.73	2.44	0.39
Per cent differences 1946-1952					I 89.59%	I 139.21%	R 61.39%
I increase R reduction							
Probability of difference due to chance*					0.159	0.041	0.219

*Any probability less than 0.05 is conventionally considered significant.

Decayed, Missing, and Filled Permanent Teeth Expressed as per Hundred Teeth.—Although the decayed, missing, and filled rates for the permanent teeth expressed as per hundred teeth and as per hundred surfaces were calculated independently of the rates found per hundred children, very little difference was noted. The 12-year-old children had a 21.79 per cent reduction when expressed as per hundred children, 19.50 per cent when expressed as per hundred teeth, and 21.10 per cent when expressed as per hundred surfaces. The 13-year-old children had reduction rates almost the same: 21.21 per cent expressed as per hundred children, 20.22 per cent per hundred teeth, and a reduction of 20.98 per cent when expressed as per hundred surfaces. The 14-year-old group had the same percentage reduction when expressed as per hundred children and per hundred teeth, 12.53 per cent and 12.97 per cent, respectively, but the percentage reduction, when expressed as per hundred surfaces, dropped to 7.62 per cent (Tables IX, X, and XI).

TABLE IX

DECAYED, MISSING, AND FILLED PERMANENT TEETH: RATE PER HUNDRED TEETH

YEAR	NO. OF EXAMS	TEETH PRESENT	TEETH EXTRACTED	TOTAL NO. TEETH CONSIDERED	DMF RATE PER 100 TEETH	PER CENT DIFFERENCES
12 Year						
1946	414	9,758	45	9,803	32.00	
1949	627	14,612	51	14,663	29.41*	–8.09%
1952	512	11,923	36	11,959	25.76*	–19.50%
13 Year						
1946	692	17,988	118	18,106	38.47	
1949	641	16,469	77	16,546	35.77*	–7.08%
1952	537	13,868	33	13,901	30.69*	–20.22%
14 Year						
1946	617	16,283	129	16,412	43.24	
1949	503	13,351	105	13,456	40.38*	–6.11%
1952	506	13,685	61	13,746	37.63*	–12.97%

*Statistically significant from base line.

TABLE X

DECAYED, MISSING, AND FILLED PERMANENT TOOTH SURFACES: RATE PER HUNDRED SURFACES

YEAR	NO. OF EXAMS	TEETH PRESENT	TEETH EXTRACTED	TOTAL TOOTH SURFACES CONSIDERED	DMF RATE PER 100 SURFACES	PER CENT DIFFERENCES
12 Year						
1946	414	9,758	45	49,015	11.42	
1949	627	14,612	51	73,315	10.53*	– 7.79%
1952	516	11,923	36	59,795	9.01*	–21.10%
13 Year						
1946	692	17,988	118	90,530	13.82	
1949	641	16,469	77	82,730	13.04*	– 5.64%
1952	537	13,868	33	69,505	10.92*	–20.98%
14 Year						
1946	617	16,283	129	82,060	15.09	
1949	503	13,351	105	67,280	14.82	– 1.78%
1952	506	13,685	61	68,730	13.94*	– 7.62%

*Statistically significant from base line.

TABLE XI

PER CENT REDUCTION (R) OR INCREASE (I) IN DENTAL CARIES EXPERIENCE RATES FOUND IN THE 1946 AND 1952 GROUPS OF 12-, 13-, AND 14-YEAR-OLD CHILDREN: RATE PER HUNDRED CHILDREN

	AGES		
	12	13	14
DMF permanent teeth	R 21.79%	R 21.21%	R 12.53%
Occlusal surface pit and fissure caries or fillings (permanent molar teeth)	R 18.53%	R 18.42%	R 11.95%
O. Surfaces free from pit and fissure caries or fillings (permanent molar teeth)	I 49.69%	I 69.39%	I 75.40%
Maxillary anterior permanent teeth (cuspid to cuspid) DF	R 24.34%	R 23.05%	R* 9.06%
Mandibular anterior permanent teeth (cuspid to cuspid) DF	R* 21.43%	R* 14.49%	R* 19.19%
Pre-carious lesions on permanent tooth surfaces	R 37.35%	R 50.91%	R 34.15%
Immune permanent dentitions	I* 89.59%	I 139.21%	R* 61.39%
DMF permanent teeth (rate per hundred teeth)	R 19.50%	R 20.22%	R 12.97%
DMF permanent tooth surfaces (rate per hundred surfaces)	R 21.10%	R 20.98%	R 7.62%

*Statistically not significant.

Summary of Findings.—In reviewing the rates in 1949 and in 1952 it is seen in Table XI that the 12- and 13-year-old children had almost exact reductions in dental caries while the 14-year-old group trailed somewhat. There was a significant reduction in the occlusal surface caries experience and a notable increase in the occlusal surface free from caries or fillings although the changes were not equal. The 12- and 13-year-old children had significant reductions in the maxillary anterior permanent teeth rates whereas the 14-year-old children did not show a statistically significant reduction. The reductions in the rates in the mandibular anterior teeth were not significant. The pre-carious lesions on the surfaces of the permanent teeth were reduced, but the reduction found in the 12-year-old children was not as great as the reduction found for children that age in 1949. The 13-year-old children were considered to have an immune permanent dentition increase which was considered to be significant. The 12- and 14-year-old children did not have significant increased rates. When considering the rates expressed as per hundred teeth and per hundred surfaces there was little difference except for the 14-year-old group, and the reductions expressed as per hundred surfaces was lower than the rate per hundred children.

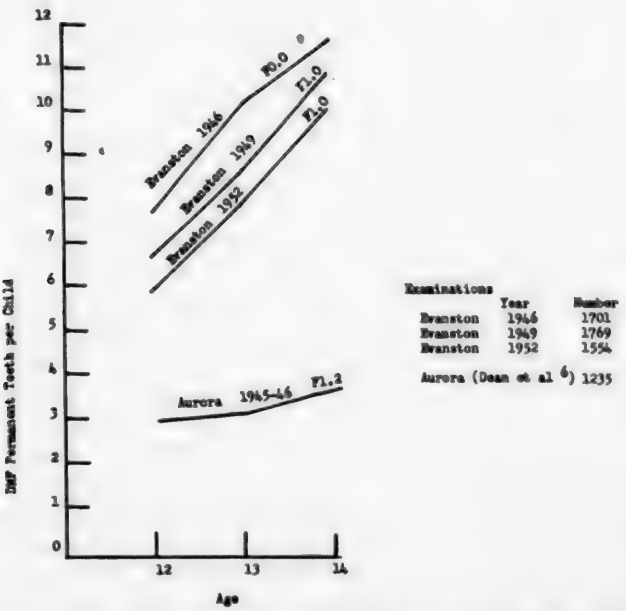

Fig. 2.—Dental caries experience of permanent teeth of Evanston and Aurora school children.

In comparing the Evanston DMF rate per child with that of Aurora, where the naturally occurring fluoride concentration is 1.2 ppm, there is a considerable difference between the rates[6] (Fig. 2). The DMF rate per child in 1952 in Evanston is 5.96, double the Aurora rate for the same age group,

2.95. The 13-year-old children of Evanston have a rate of 7.95, which is approximately 2.57 times greater than the Aurora rate of 3.09; the 14-year-old group in Evanston have a rate of 10.19, which is 2.79 times the Aurora rate of 3.64 for the same aged students.

Since these data provide the caries rate during five years of fluoridation and indicate a decrease in dental caries experience of approximately 20 per cent or a 4 per cent reduction for each year of fluoridation, the Evanston rate of dental caries should be somewhere near the Aurora rate in 1957, which is after ten years of fluoridation, providing there is no occurrence of a leveling effect before that time. It will always be difficult, if not impossible, to make exact comparisons between different studies because methods of examinations cannot be exactly standardized and variations usually are present.

DISCUSSION

When sodium fluoride was added to the water in 1947 these 12-, 13-, and 14-year-old children were 7, 8, and 9 years of age, respectively, with the crowns of their canines, premolars, and second molars fully calcified. For from one to five years prior to eruption, the enamel of the crowns of these teeth was exposed to tissue fluids containing the F ion. It was logical to assume that the greatest reduction in dental caries probably would be found in the 12-year-old group as the canines and second molars are usually in the final stages of eruption at about the twelfth year and thus could experience the longest pre-eruptive period after fluoridation. Although the crowns of the permanent dentition were formed at the time fluorine was placed in the water, these teeth were exposed to tissue fluids containing fluorine during the pre-eruptive and eruptive stages. The dental caries reduction found in the 12- and 13-year-old group being approximately equal may be attributed to the fact that at the time of examination the canines and second molars of some of the 12-year-old children had not yet erupted; we consider a child of 11 years and 6 months to be in the 12-year-old group. These particular teeth of the 13-year-old children were erupted but may not have been exposed to the oral environment a sufficient length of time for caries to develop. Boyd and Wessels[7] observed carious lesions on one-half of the occlusal surfaces of second molars of forty-nine girls and boys within one year after eruption and on three-fourths of the surfaces within eighteen months.

While considering the occlusal surfaces with and without pit and fissure caries or fillings, we noted a decrease in the caries experience rates and an increase in the number free from carious experience; however, the changes were not equal. It is felt that until the rates are expressed as per hundred teeth or per hundred surfaces, noting the total number of surfaces present, the imbalance will remain. However, the changes in rates were considered significant.

The caries experience reductions in the maxillary and mandibular anterior teeth, although quite consistent, were not considered to be too important. The numbers of affected teeth in both the maxillary and mandibular sections

were so small that only the reduction in the maxillary anterior rates of the 12- and 13-year-old children were considered significant. The reduction found in the 14-year-old group probably could happen from year to year as a normal fluctuation. None of the changes affecting the mandibular teeth were significant.

The rates for the permanent tooth surfaces with pre-carious lesions were somewhat baffling for the 12-year-old group. There was a reduction from the 1946 rate, but the 1952 rate was higher than the rate found in 1949. The increase from 1949 was considered to be significant as was the decrease of the 1952 rate from 1946.

The cases considered to be immune to dental caries at the time of examination in 1952 indicated an increase of 89.59 per cent for the 12-year-old children, 139.21 per cent for the 13-year-old students, and a reduction of 61.39 per cent for the 14-year-old children. The increase for the 13-year-old pupils was considered to be significant. The fact that the changes for the 12- and 14-year-old groups were not significant was attributed to the small number of immune cases found each year.

SUMMARY

In considering the changes in decayed, missing, and filled rates, the DMF rates per hundred children, per hundred teeth, and per hundred surfaces all give about the same percentage reduction.

The maxillary anterior teeth decayed and filled rates were reduced about 23 per cent for the 12- and 13-year-old children, while the reduction for the 14-year-old students of 9.06 per cent was not considered significant. The mandibular anterior teeth decayed and filled rate reductions were not significant.

The increase in immune permanent dentitions was statistically significant only for the 13-year-old groups, and here an increase of 139.21 per cent was noted. However, the increase was only 1.42 cases per hundred children when 1952 was compared to 1946.

The percentage reduction change in the dental caries rates for the 12-, 13-, and 14-year-old children after exposure to fluoridated water from fifty-nine to seventy months was approximately 18.51 per cent for the combined ages. This reduction is considered to be statistically significant. The reduction to date appears to be approximately 4 per cent for each year of fluoridation.

The authors wish to express appreciation for the cooperation given them by the members of the Evanston Dental Caries Study, Drs. Jelinek, Neff, and Greco; Miss Joann Johnson, x-ray technician, and Mrs. M. Seery, secretary. The generous assistance of Mrs. M. McManus, Evanston Health Department, and Miss B. Ferguson, secretary, Zoller Dental Clinic, is particularly appreciated.

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RESULTS OF AN EIGHT-YEAR STUDY OF THE EFFECTIVENESS OF CARBOHYDRATE RESTRICTION IN REDUCING SALIVARY LACTOBACILLUS COUNTS

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CONSISTENTLY high counts of salivary lactobacilli have been found to be closely related to concurrent dental caries activity. Consistently low counts of salivary lactobacilli have been found to be associated with little or no concurrent activity of dental caries.

Periodic counts of salivary lactobacilli have been used for some years by the Caries Control Laboratory of the University of Michigan School of Dentistry in the diagnosis of dental caries activity. Lactobacillus counts have also been used to determine the effectiveness of a dietary treatment (the elimination of sucrose and the reduction of other carbohydrates) in arresting dental caries activity.

In October, 1944, the Ohio State University College of Dentistry established a Caries Control Laboratory patterned closely after the Michigan Laboratory. The technic of making salivary lactobacillus counts has been kept identical with that used at Michigan, and so the results of the two laboratories are comparable. The recommended low carbohydrate diets used in the Ohio Laboratory have been varied somewhat from those originally recommended at Michigan, but both laboratories have consistently recommended the elimination of sucrose from the diet and the reduction of other carbohydrates for periods of several weeks.

Jay[1] published the results of the periodic restriction of carbohydrates on the oral lactobacillus counts as obtained in the Michigan Caries Control Laboratory. We followed Jay's report with a report of the data obtained in the Ohio Caries Control Laboratory during a period of three years.[2] The present report is a supplement to the 1948 report, and includes data from the Ohio Laboratory records from October, 1944, to June, 1953.

Briefly, the procedure for diagnosis and treatment of active dental caries has been as follows:

Two stimulated saliva specimens are collected on consecutive days before the subject eats or brushes the teeth, and lactobacillus counts are made in the manner developed in the Michigan Laboratory. A patient who has a salivary lactobacillus count of over 10,000 per cubic centimeter of saliva is regarded as caries active. For this patient it is recommended that carious areas be repaired immediately. Then the patient is instructed to follow, for a period of two weeks, a diet free of sucrose and low in other carbohydrates (as near as possible to 100 Gm. of carbohydrate a day). This diet is referred to as Plan I

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diet, and is free of bread, potatoes, and high carbohydrate fruits and vegetables, as well as of sucrose. Careful adherence to this diet will nearly always result in a reduction of the salivary lactobacillus count to practically zero. At the end of a two-week period on Plan I diet, a count of salivary lactobacilli is made, and the patient is instructed to change to Plan II diet. This is Plan I diet with the addition of some bread and some fruits and vegetables with a higher content of carbohydrate. At the end of two weeks of Plan II diet, another salivary lactobacillus count is made. If the count has remained low, the patient is instructed to change to Plan III diet. This consists of Plan I diet with the addition of bread, unrestricted fruits and vegetables, and the equivalent of one teaspoonful of sugar per day, if desired. After a two-week period of Plan III diet, the salivary lactobacillus count is determined, and if it is low, the patient is allowed to change to Plan IV diet. This is a nearly unrestricted diet. However, the patient is instructed to keep sugar consumption low if he wishes to avoid a subsequent increase in the salivary lactobacillus count with its resultant resumption of dental caries activity.

It is the intention of this Laboratory to follow each patient with periodic lactobacillus counts for a period of at least two years after the initial dietary treatment has been completed. Any increase in the counts is followed by recommendations to the patient to restrict sugar consumption.

DATA

Table I is a compilation of the records of the Dental Caries Control Laboratory of the Ohio State University College of Dentistry, from October, 1944, to June, 1953, of the salivary lactobacillus counts of 1,089 individuals. With the exception of a small number of patients from the school's clinic, these individuals were patients of private dentists practicing in Ohio and in surrounding states. Laboratory reports of the lactobacillus counts were sent to the dentists, and diets were prescribed through the dentists.

Of the 1,089 individuals examined, 278 (26 per cent) were found to have a low salivary lactobacillus count for the two initial specimens. In subsequent saliva examinations, 101 of this number were found to have lactobacillus counts increased to over 10,000 per cubic centimeter of saliva. These individuals with increased counts were advised to undertake dietary treatment.

Of the 1,089 individuals examined, 811 (74 per cent) were found to have over 10,000 lactobacilli per cubic centimeter of saliva in the two intial specimens. These individuals were regarded as caries active, and dietary treatment was recommended.

Of the 912 (811 plus 101) individuals who were found to need dietary treatment, 531 have completed two weeks supposedly following Plan I diet. The salivary lactobacillus counts made on saliva specimens at the end of two weeks following Plan I diet indicated that 410 (77 per cent) of the patients had greatly reduced their lactobacillus counts (to 0 to 5,000 per cubic centimeter). One hundred twenty-one (23 per cent) had not succeeded in reducing the lactobacillus count to below 5,000 per cubic centimeter during the first two weeks. Some of this number discontinued treatment at this point; others later showed reduced counts and proceeded with the dietary treatment.

TABLE I

EFFECT OF DIETARY TREATMENT ON THE SALIVARY LACTOBACILLUS COUNTS OF PATIENTS TREATED THROUGH THE DENTAL CARIES CONTROL LABORATORY OF OHIO STATE UNIVERSITY (THE FIGURES IN PARENTHESES ARE THOSE PUBLISHED BY JAY[1] FOR THE MICHIGAN LABORATORY)

	TOTAL NUMBER	PATIENTS WITH LOW LACTOBACILLUS COUNTS		PATIENTS WITH HIGH LACTOBACILLUS COUNTS		PATIENTS WHO HAD HIGH LACTOBACILLUS COUNTS WHICH WERE LATER REDUCED TO LOW COUNTS FOR A PERIOD OF 6 MO. OR MORE
		NUMBER	PER CENT	NUMBER	PER CENT	
Total number of individuals examined	1,089	278	26	811	74	
Low counts which later became high	101					
High counts: Dietary treatment undertaken, Plan I supposedly followed for at least 2 wk.	531	410	77 (83.4)	121	23	
After completion of Plan II	376	318	85 (81.6)	58	15	1
After completion of Plan III	306	225	74 (73.4)	81	26	2
After completion of at least 2 wk. on Plan IV (unrestricted diet)	202	142	70 (81.2)	60	30	5
Checked after 6 mo. on Plan IV	126	91	72	35	28	14
Checked after 1 yr. on Plan IV	75	53	71	22	29	10
Checked after 2 yr. on Plan IV	34	25	74	9	26	3
Checked after 3 or more yr. on Plan IV	23	14	61	9	39	2

There are 376 patients who have completed two weeks following Plan II diet. Of this number, 318 (85 per cent) had continued low salivary lactobacillus counts at the end of the two-week period. Fifty-eight (15 per cent) experienced an increase in the lactobacillus count at this time. One of these patients later showed a decreased count and continued with dietary treatment.

There are 306 individuals who have completed two weeks following Plan III diet. Of these, 225 (74 per cent) had continued low salivary lactobacillus counts at the end of the two-week period. Eighty-one (26 per cent) had increased counts at the end of this period. Two of the eighty-one returned to the Plan II diet and later showed low counts and continued dietary treatment.

There are 202 patients who have completed at least two weeks following Plan IV diet. This is a diet which is unrestricted except that the patient is advised to keep sugar consumption low and infrequent. Of the 202, 142 (70 per cent) had continued low lactobacillus counts at the end of the two-week period. Sixty (30 per cent) had increased counts at this time. Of the sixty, five returned to the Plan II diet, reduced the count, and maintained a reduced count for at least six months thereafter.

There have been 126 patients whose salivary lactobacillus counts have been determined after six months of following Plan IV diet. Ninety-one (72 per cent) of these had continued low counts. Thirty-five (28 per cent) had increased counts. Of the thirty-five, fourteen of the patients returned to Plan II diet, reduced the salivary lactobacillus count, and maintained the reduction for at least six months thereafter.

There are seventy-five individuals whose salivary lactobacillus counts have been determined after one year on Plan IV. Fifty-three (71 per cent) had continued low counts. Twenty-two (29 per cent) had increased counts one year after the close of the treatment. Of this number, ten returned to Plan II, reduced the count, and maintained a low count for at least six months thereafter.

There are thirty-four patients whose salivary lactobacillus count has been determined at the close of two years following dietary treatment. Of these, twenty-five (74 per cent) had maintained a low count. In nine (26 per cent) of the thirty-four individuals, the lactobacillus count had increased. Three of these returned to Plan II diet, reduced the count, and maintained the reduced count for at least six months thereafter.

There are twenty-three individuals whose lactobacillus count has been determined after three or more years following dietary treatment. Of these, fourteen (61 per cent) had maintained a low count. Nine (39 per cent) had experienced an increase in the lactobacillus count. Of the nine, two returned to Plan II diet, reduced the count, and maintained the low count for at least six months thereafter.

DISCUSSION

The data in Table I indicate the results obtained by the Ohio State University Caries Control Laboratory in the dietary treatment for reducing salivary lactobacillus counts as a means of controlling dental caries activity. The results obtained by Jay[1] are given in parentheses. The results of the Ohio Laboratory compare favorably with those published by Jay for Michigan. Jay's data indicate that the Michigan Laboratory had a somewhat higher percentage of successes in reducing the salivary lactobacillus count by the initial two-week dietary period. The Ohio data indicate a higher percentage of low salivary lactobacillus counts among the individuals completing two weeks of the Plan IV diet. These differences probably are of no significance.

In every case where careful investigation of the diet of patients under treatment has been possible, we have found that failure of the reduction of the salivary lactobacillus count during the dietary period was associated with the failure of the patient to refrain from eating sucrose.

The relatively small number of cases followed for a period of more than six months is the result of the patients' voluntarily discontinuing the collection of saliva specimens.

SUMMARY

The Ohio State University Dental Caries Control Laboratory has, over a period of eight years, made salivary lactobacillus counts on 1,089 patients. Of this number, 811 individuals had over 10,000 lactobacilli per cubic centimeter of saliva at the time of the initial counts. Two hundred seventy-eight had salivary lactobacillus counts of less than 5,000. Of these 278, 101 subsequently had increased counts. Of the caries-active individuals, 410 completed two weeks of the Plan I diet with low counts. Of these, 318 completed Plan II with low counts. Of the 318, 225 completed Plan III with low counts. One

hundred forty-two continued to have low counts after two weeks of Plan IV diet. Ninety-one of 126 individuals checked had low counts after six months on Plan IV. Fifty-three of seventy-five individuals checked had low counts after one year on Plan IV diet. Twenty-five of thirty-four individuals checked had low counts after two years on Plan IV diet. Fourteen of twenty-three individuals checked had low counts after three or more years on Plan IV. The results compare favorably with those published by Jay.

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DENTAL CARIES AND GINGIVITIS IN TEL AVIV, ISRAEL

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INTRODUCTION

THE only previous report on the dental health of children living in Israel was made by Mansbach in 1936. This report showed a very low caries attack rate among children living in the collective settlements when compared to children of the same age and ethnic origin living in neighboring farms and in the cities. No additional surveys were conducted subsequently because of the heavy treatment load on the school dentists (one part-time dentist for 3,000 to 4,000 pupils) and because of the intervening wars.

The dental situation in Israel is unique in many respects. The mass migration of entire communities and their resettlement in homogenous ethnic groups afford the investigator an excellent opportunity for comparative studies in the prevalence of dental caries and gingivitis in entire communities with entirely different food habits and cultural patterns. In addition, in the city of Tel Aviv, approximately 90 per cent of all school children are not only examined but also treated by school dentists. The uniformity of dental treatment affords the investigator a controlled situation this respect.

A country-wide survey of the prevalence of dental caries, gingivitis, and other dental disorders is now in progress under the sponsorship of the Department of Health of the state of Israel. This report is a preliminary survey made on 7,454 of the approximately 50,000 school children living in Tel Aviv. It is published in order to aid in the orientation of other surveys, since the tabulation of the final nationwide data will require a considerable period of time.

METHOD AND MATERIALS

This report is based on the clinical examination of 7,454 school children 13 to 17 years of age living in Tel Aviv, Israel. Caries experience was recorded and assessed as DMF rates, and gingivitis as the P-M-A (papillary, marginal, attached) index.

Age was computed according to grade in school rather than by direct questioning of each individual. The age of each class was relatively homogeneous and was estimated by averages made from the records in the Department of Education.

DMF Rate.—The fairly well-standardized technic of evaluating dental caries experience as described by Klein and Palmer[3] was used in this survey. The number of decayed (D), missing (M), including teeth indicated for extraction, and filled (F) teeth was recorded for each child and the total computed as the DMF rate per child per age group. All examinations were made using mouth mirror and explorer under good lighting conditions.

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Since the major source of error in epidemiologic surveys is the divergence in observations among different examiners, the 10 school dentists participating in this survey were carefully trained in the technic of examination on small groups until their observations were considered reliable and reproducible. Fifteen classes (433 children) were examined twice to check the accuracy and consistency of each examiner. Only 1 of the 10 examiners deviated more than 15 per cent from the mean of this sample.

During the course of the survey, examinations were performed by one dentist while another looked on to check his results. The roles were reversed in the next class. All recordings were made by an experienced dental nurse.

P-M-A Index.—Gingivitis was assessed and recorded according to the method described by Massler, Schour, and Chopra.[6] Here again, each examiner was carefully trained in the technic and checked frequently until his observations were considered reliable and reproducible. The number of papillary (P), marginal (M), and attached (A) gingivae affected per child was recorded, and the total was computed as the average P-M-A index per age group.

Other dental conditions observed and recorded are listed in Table IV.

FINDINGS

DMF Rate.—Table I lists the DMF rate of this sample of children living in Tel Aviv and compares the findings with children living in New Zealand[2] and children living in Philadelphia, Pennsylvania.[1] The differences among these groups are striking. The caries experience among the Israeli children was approximately one-half that reported for the children of Philadelphia and approximately one-third of that reported for New Zealand children.

TABLE I

CARIES EXPERIENCE IN 7,454 SCHOOL CHILDREN LIVING IN TEL AVIV COMPARED WITH THAT IN SCHOOL CHILDREN IN PHILADELPHIA AND IN NEW ZEALAND

AGE IN YEARS	NUMBER OF CHILDREN EXAMINED	AVERAGE NUMBER OF TEETH AFFECTED PER PERSON					
		TEL AVIV				PHILADELPHIA, PA.*	NEW ZEALAND†
		D	M	F	DMF	DMF	DMF
13	2,724	0.77	0.06	1.72	2.55	5.8	8.87
14	3,461	0.87	0.09	2.10	3.06	8.1	10.01
15	614	1.15	0.10	2.84	4.09	8.2	
16	449	1.19	0.18	3.07	4.44	9.6	
17	206	0.84	0.09	4.27	5.20	10.0	
Total	7,454						

*Cohen and Massler, 1952.
†Fulton, 1951.

A further breakdown of the DMF rate into its separate components shows also striking differences in the tooth mortality (M) and filling (F) rates in Tel Aviv, Philadelphia, and New Zealand. Table III shows, for the 14-year age group, a tooth loss of 108 teeth per 100 children in Philadelphia, forty-one in New Zealand, and only nine in Tel Aviv. It was observed that the majority of extractions in Tel Aviv occurred among the immigrant children. The tooth mortality among the native children is therefore undoubtedly even lower.

P-M-A Index.—Table II indicates the P-M-A index of gingivitis for the children of Tel Aviv. This index is much lower (approximately one-fifth) than that reported for children living in Philadelphia.[5] This difference is highly significant and could not be due to differences in examining technic since the latter was checked by Dr. Isaac Schour during his visit in Israel when he participated in the training of these examiners.

TABLE II

GINGIVITIS EXPERIENCE IN 7,454 SCHOOL CHILDREN LIVING IN TEL AVIV

AGE IN YEARS	NUMBER OF CHILDREN EXAMINED	AVERAGE NUMBER OF GINGIVAL UNITS AFFECTED PER PERSON				
		TEL AVIV				PHILADELPHIA, PA.*
		P	M	A	P-M-A	P-M-A
13	2,724	0.42	0.31	0.02	0.75	4.9
14	3,461	0.43	0.34	0.03	0.80	4.9
15	614	0.44	0.46	0.03	0.93	5.0
16	449	0.56	0.63	0.01	1.20	5.1
17	206	0.93	0.46	0	1.39	5.1
	7,454					

*Massler, Cohen, and Schour, 1952.

TABLE III

COMPARISON OF DMF RATES IN 14-YEAR-OLD CHILDREN LIVING IN TEL AVIV, PHILADELPHIA, AND NEW ZEALAND

	TEL AVIV	PHILADELPHIA, PA.	NEW ZEALAND
DMF	3.06 (100 %)	8.10 (100 %)	10.01 (100 %)
D	0.87 (28.4%)	3.40 (42.0%)	0.99 (9.9%)
M	0.09 (2.9%)	1.08 (13.3%)	0.41 (4.1%)
F	2.10 (68.7%)	3.40 (42.0%)	8.61 (86.0%)

Dental Anomalies.—Table IV lists the incidence of various dental anomalies as observed during these examinations. The incidence of malocclusions is much lower in these children than generally reported in the United States.[7] The incidence of enamel hypoplasia is similarly much less than generally reported.[8] The low incidence of mottling is consistent with the chemical analysis of the thirty-five wells in Tel Aviv, which showed a fluoride content varying from 0.3 to 1.2 ppm.

TABLE IV

DENTAL ANOMALIES IN 7,454 SCHOOL CHILDREN LIVING IN TEL AVIV, ISRAEL

CONDITION	CHILDREN AFFECTED (%)
Malocclusion	21
Enamel hypoplasia	0.7
Mottled enamel	2.6
Peg teeth	0.6

DISCUSSION

The striking difference in caries experience of the children living in Philadelphia and New Zealand (Table I) could not be due to the fluoride content of the water supply since the city uses thirty-five different wells with a fluoride

content ranging from 0.3 to 1.2 ppm. It is more likely that the relative absence of sweets in Tel Aviv may account for this difference. The average per capita consumption of refined sugar is 30 pounds per year, compared to the more than 120 pounds consumed in the United States per person per year. The abundance of vegetables in the Israeli dietary may be another factor.

Possible Basis for Low Prevalence of Gingivitis in School Children in Tel Aviv.—The very low P-M-A index in Tel Aviv could not be ascribed to superior oral hygiene, since the oral hygiene program for the school children living in Philadelphia is one of the best in the United States,[5] while the use of a toothbrush by the children of Tel Aviv is considerably less intensive. The physical character of the diet, particularly the superior detergent action and abrasive character of the Israeli dietary, may be a basic reason for the lower gingivitis rate in Tel Aviv.

There did not appear to be any consistent relationship between the prevalence of caries and of gingivitis.

Tooth Mortality Rates.—The tooth mortality rate is a good index to the effectiveness of any dental health service, private or public.[2] The tooth mortality rate at the age of 14 years in the children of New Zealand is less than one-half of the rate in the children of Philadelphia, in spite of the fact that the caries attack (DMF) rate is higher in the children of New Zealand (Table III). The universal school dental health service program in New Zealand is apparently effective in preventing excessive tooth loss in spite of the high caries attack rate. In Philadelphia, on the other hand, the children receive treatment chiefly from private practitioners.

The tooth mortality rate in Tel Aviv is lowest, one-tenth of that in Philadelphia, and one-fifth of that in New Zealand. Tel Aviv is fortunate in having both a low caries attack rate and a school treatment program. The combination leads to an extraordinarily low rate of tooth loss.

A better method of comparing the effectiveness of the dental service is to equate the caries attack rate in the three areas and to calculate the percentage of teeth lost on the basis of the number attacked by caries (DMF equals 100 per cent) (Table IV). This reveals that only 2.9 per cent of the teeth attacked by caries are lost in the children of Tel Aviv, 4.1 per cent of the teeth attacked are lost in New Zealand, while in Philadelphia 13.3 per cent of the teeth attacked by caries are lost by extraction.

Filling Rate.—The percentage of teeth attacked by caries, which are filled, is another index as to effectiveness of the dental care program. In this respect New Zealand, with its universal school service program, ranks highest with 86 per cent of the carious teeth being filled in children aged 14. In Tel Aviv, 68.7 per cent of the decayed teeth are restored, most of these by the school dentist, but many by private practitioners.

In this respect the dental health program in Tel Aviv is not as effective as that in New Zealand. In Tell Aviv 28.4 per cent of the teeth of children aged 14 were not repaired, compared to only 9.9 per cent not repaired in New Zealand (Table IV). As soon as more dentists become available to the school service program in Israel, this deficiency should be corrected.

In Philadelphia, only 42 per cent of the carious teeth were filled (and 42 per cent were unfilled), all by private practitioners. This filling rate is probably even lower in communities which do not have the excellent school dental health program sponsored by the Board of Education of the city of Philadelphia.

Caries Rate in Yemenite Children.—During the course of these examinations it became evident that the children born and raised in Yemen had a much lower DMF rate than the children living in Tel Aviv. Dr. Mandel, a school dentist in Tel Aviv, therefore examined ninety-four Yemenite children (ages 12 to 14 years) living in Rash-Ajin (Fountainhead). He found the DMF rate to be 0.58, about one-fifth that in Tel Aviv (DMF, 3.0). The P-M-A index (1.6), on the other hand, was approximately twice as high as in Tel Aviv (0.80). Although the sample is very small, these differences are so striking as to be evident clinically even after superficial examination.

The reason for the lower DMF rate in the Yemenite children is not clear. There is perhaps a strong genetic factor which bears further investigation. The higher P-M-A index is more easily explained, since calculus is far more evident among the Yemenite children. These people live in a Bedouin civilization which has not changed significantly during the past 1,000 years. Toothbrushes are unknown.

SUMMARY AND CONCLUSIONS

The caries (DMF) experience and gingivitis (P-M-A) experience of 7,454 children 13 to 17 years of age living in Tel Aviv, Israel, were investigated and compared with those reported for New Zealand and Philadelphia. For 14-year-old children the DMF index was 3 in Tel-Aviv, 8 in Philadelphia, and 10 in New Zealand. For the same age group the P-M-A index was 0.8 in Tel Aviv and 4.9 in Philadelphia.

The reason for the low caries rate in Israel may be at least in part the result of the relatively low sugar intake. The low gingivitis rate may be in part related to the more detergent physical character of the diet in Israel.

Analysis of the tooth mortality rate and the number of fillings indicates that the school dental service program as currently present in New Zealand and in Israel is superior to the private care rendered for children in the United States, inasmuch as the percentage of teeth filled is much higher in New Zealand and Israel and the number as well as the percentage of teeth lost by extraction is much less in New Zealand and Israel than in Philadelphia. The extraordinarily low tooth mortality rate in Israel (9 teeth lost per 100 children at age 14 compared to 108 teeth lost per 100 children living in Philadelphia) is the result of a relatively low caries attack rate and a good school service program.

Further studies now in progress on the dental health status of Israeli children of particular ethnic groups are likely to throw new light on the problem of preventing dental decay and gingivitis.

The findings in this survey show that a public dental health service which gives dental treatment to every pupil as an integral part of his educational program is very effective in reducing the tooth mortality rate.

The advice and encouragement of Drs. Isaac Schour and Maury Massler are acknowledged with appreciation.

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THE PRODUCTION OF ERYTHEMA OF THE SKIN BY EXPOSURE TO X-RAY RADIATION

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ONE manifestation of overexposure of the skin to x-ray radiation employed diagnostically is the appearance of an erythema similar to sunburn. This paper presents the experimental results of a study which was completed to determine the amount of radiation from a dental x-ray machine that is required to produce an erythema of the skin on the flexor surface of the forearm.

There are many references in dental literature which state that an exposure of 1,200 milliampere seconds* at a target-skin distance of 8 inches will cause an erythema of the skin; therefore, no single area of skin should be exposed to more than one-half of the erythema dose, or 600 Ma.S., in any two-week period. To test the validity of these statements, circular areas of skin, 1⅛ inches in diameter, were exposed on the flexor surface of the forearms of 20 male volunteers of college age.

In the beginning phase of this investigation, exposures were initially of short duration and then were gradually lengthened to approach the exposure time necessary to produce a clearly defined pink area on the forearm. The smallest exposure found to produce an erythema was 425 Ma.S., although many subjects did not experience an erythema until they had received 550 to 600 Ma.S. of exposure to x-ray radiation. After the approximate magnitude of the exposure required to produce erythema had been determined, a second series of tests was performed on nineteen additional male volunteers of college age in an effort to determine more exactly the amount of radiation from a dental x-ray machine that is required to produce an erythema of the skin on the flexor surface of the forearm.

Each subject was given 600 Ma.S. of x-ray exposure, which was administered to a circular area on the flexor surface of one forearm. This area was observed again in twenty-four hours. If an erythema had appeared, a similar area on the flexor surface of the other arm was given 10 per cent less exposure, or 540 Ma.S. If no erythema had appeared, the other arm was given 10 per cent more exposure, or 660 Ma.S. The initial appearance of the erythema was determined by the subject. The results of the experiments with these nineteen individuals are listed in Table I.

Six of the nineteen subjects did not cooperate to the extent of permitting the second arm to be exposed. However, even the exposure of one arm provided

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*"Milliampere seconds" (abbreviated Ma.S.) is the unit of x-ray exposure or dose which is the product of the number of milliamperes of current flowing through the x-ray tube and the duration of the exposure expressed in seconds.

some useful information. If an erythema of the skin resulted from a 600 Ma.S. exposure, it can be assumed that a similar reaction would result also from a 660 Ma.S. exposure. If no erythema of the skin resulted from a 600 Ma.S. exposure, it can be assumed that no reaction would result from an exposure of 540 Ma. S.

TABLE I

REACTION OF THE SKIN TO X-RAY RADIATION

SUBJECT	540 MA.S.	600 MA.S.	660 MA.S.
1		+	
2		−	
3	−	+	
4	+	+	
5	+	+	
6	+	+	
7	+	+	
8		+	
9	+	+	
10		−	+
11	−	+	
12		−	−
13	−	+	
14	+	+	
15		−	+
16		−	
17		+	
18		+	
19	−	+	

Not more than 540 Ma.S. of exposure was necessary to produce an erythema in six of fifteen subjects or 40 per cent. Not more than 600 Ma.S. of exposure was necessary to produce an erythema in fourteen of nineteen subjects (73.7 per cent). Not more than 660 Ma.S. of exposure was necessary to produce an erythema in sixteen of seventeen subjects (94.2 per cent).

The erythema appeared in two to eight hours after the skin was exposed and remained visible until the third to fifth day, at which time tanning or pigmentation replaced the erythema. The phase of pigmentation was of four to eighteen weeks' duration. Alopecia (loss of hair) did not develop at any time in the test sites.

A modern dental x-ray unit, operated at 65 KVP and 10 Ma., was used throughout this study. The inherent filtration of the unit was approximately 1 mm. of aluminum. No additional filtration was employed. The x-ray unit delivered 165r per minute in air as measured opposite the tip of the pointed plastic cone at a distance of 7½ inches from the focal spot of the x-ray tube. The half-value layer of the radiation was 0.7 mm. of aluminum.

The output of an x-ray machine can be measured in air at any specified position by means of a suitable measuring device. If the body of a patient or phantom is introduced into the beam, so that the skin or the surface of the phantom is at this same position, the number of roentgens per minute at this position is increased, because of a scattering of the radiation back from the underlying material. This back-scattering varies with the quality of the radiation, the diameter of the x-ray beam, and the depth of scattering material

present.[1] The skin dose is the amount of radiation the skin receives and is the sum of the radiation from the x-ray machine measured in air and the back-scatter.

From measurements made on a water-filled phantom (6 inches by 6 inches by 3½ inches deep), it was found that 100 r measured in air equals a skin dose of 104 r when an x-ray beam 1⅛ inches in diameter is employed, and 109 r when the beam is 4 inches in diameter. At a focal-skin distance of 7½ inches, it was found that 600 Ma.S. produced an erythema of the skin on the flexor surface of the forearm in about three-quarters of the subjects. For the x-ray machine used in this study, a 600 Ma.S. exposure equaled 165 r measured in air, or a skin dose of 172 r when the diameter of the beam was limited to 1⅛ inches. With the 4-inch diameter beam, the skin dose would be 180 r.

Belisario,[2] Erskine,[3] and Herrmann[4] have found that parts of the body ordinarily covered with clothing are more sensitive to x-ray radiation than exposed areas like the face or hands, with their thicker skin. In the present study, only the skin on the flexor surface of the forearm was tested. Therefore, no experimental information is available regarding the quantitative difference in sensitivity to x-ray radiation between the skin on the face and the flexor surface of the forearm. However, it can be reasoned that a 400 Ma.S. exposure which produced no reaction on the flexor surface of the forearm could likewise be tolerated by the less sensitive skin of the face. When the x-ray machine involved in these tests is used for dental radiography, it exposes a circular area of skin of about 4 inches in diameter on the face. It had been determined experimentally that the amount of back-scattering increased about 5 per cent when the diameter of the irradiated area was increased from 1⅛ to 4 inches. The increase in the skin dose on the face incurred through the use of the large diameter x-ray beam would nullify partially the factor of the skin on the face being less sensitive to x-ray radiation than on the flexor surface of the forearm. Any remaining portion of this factor involving the variation in sensitiveness to x-ray of the two skin areas can be considered a factor of safety to allow for idiosyncrasy of the patient to radiation and mechanical variations among dental x-ray machines. Therefore, it is suggested that no one area of the face be exposed at a 7½ inch target-skin distance to more than 400 Ma.S. in any two-week period. No experimental evidence has been presented regarding the time element of a "two-week period." This period of time is advocated here, just as it is throughout the dental literature, because no new evidence has been offered to sustain or refute this commonly accepted period of time.

SUMMARY

Experiments performed on human skin irradiated with a modern dental x-ray machine indicate that an erythema of the skin on the flexor surface of the forearm can be produced by an exposure of 600 Ma.S. ("165 r in air"). Such exposure produced an erythema on three of four patients. The least exposure required to produce an erythema on anyone in these tests was 425 Ma.S. Recognizing that the skin of the face is less sensitive to x-ray radiation than

the skin on the flexor surface of the forearm and utilizing this difference in sensitivity as a safety factor, it has been recommended that no single area of the face be exposed at a 7½ inch target-skin distance to more than 400 Ma.S. ("110 r in air") in any two-week period.

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SIMPLIFIED METHODS FOR DETERMINATION OF CALCIUM AND MAGNESIUM IN THE SALIVA

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THIS article describes the application to saliva of two analytic methods: the titration of calcium and magnesium with disodium ethylene diamine tetraacetic acid, and the colorimetric determination of magnesium by the titan yellow procedure. Observations made during the work which seem to be of more general interest are also reported.

I. Calcium and Magnesium Determination by Complexone Titration

Calcium, magnesium, and other metals are firmly bound in un-ionized complexes by certain organic compounds which have been called complexones. Certain dyes have a similar, although weaker, metal-binding capacity. If the color of such a dye is distinctly different from that of its complex with a metal, it can be used as an indicator for titration of the metal ion in question with a complexone.

Techniques based upon this principle have been rapidly developed during the last few years. For determination of the alkaline earth metals, disodium ethylene diamine tetraacetate (EDTA) has been applied most often; a basic paper on this subject is that of Biedermann and Schwarzenbach.[2]

In the biologic field, methods have been evolved to determine calcium in plasma[3] and in serum, urine, and spinal fluid[15] by titration with EDTA, using murexide (ammonium purpurate) as indicator. Another technique uses Eriochrome Black T as indicator for Mg ions. This dye shifts from red to blue when all Mg ions have been bound by the EDTA titration solution. It can hardly be used for titrations of Ca alone, because its color shift is then too gradual and indistinct. If Ca and Mg are present simultaneously, however, Ca is bound first by the EDTA, and a sharp end point is obtained in the Mg range. The total amount of Ca and Mg can thus be determined; if Mg is determined in a separate sample after the Ca has been precipitated as the oxalate, Ca is obtained as the difference. Such methods have been applied mainly in water analysis,[5, 11] but also for determinations in biologic fluids.[9]

A third method, used for ultra-micro estimations on serum,[31] involves the same determination of Ca and Mg, followed by the separate determination of Mg by a special colorimetric method[27]; Ca is obtained as the difference.

ORIGINAL INVESTIGATION

Various modifications of the methods outlined previously were tested with reference to suitability of calcium and magnesium analysis in the saliva. The

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technique finally adopted was found to be about as accurate as, and much more convenient than, the method[29] previously used by the present author, the principles of which were: precipitation of Ca as the oxalate, conversion into the carbonate by ignition, and titration of the carbonate with hydrochloric acid. Lazarev[25] described this procedure as the most accurate of established techniques for calcium analysis.

The use of murexide as an indicator for direct calcium titration was found impracticable, because the color shift was much too weak and indistinct to the investigator's eye; nor were efforts with spectrophotometric reading of the color shift successful. The work was therefore concentrated on the titration of Ca and Mg with Eriochrome Black T as indicator, and the separate determination and deduction of Mg.

The EDTA titration was thoroughly tested with known salt solutions before being applied to the saliva. The general procedure is described in the following:

The EDTA titration must be performed in an alkaline buffer of pH about 8 to 10, since the indicator Eriochrome Black T is also a pH indicator, having a wine red color below pH 6.3 and an orange color above pH 11.5. The indicator is sensitive not only to Ca and Mg ions but also to a great number of other cations. [2, 5] None of these is, however, present in the saliva except as traces, e.g., of Cu and Fe.

An important observation was made when different concentrations of Mg standard in the range 0.1 to 2.0 mM per liter were titrated: the EDTA consumption is not proportional to the Mg concentration, being comparatively higher at lower concentrations. The reason for this phenomenon seems to be indicated by the fact that a higher indicator concentration gave a greater deviation from proportionality. The indicator Eriochrome Black T probably binds an appreciable fraction of a low Mg concentration.[2] Since the deviation was found to be linear, at least up to 2 mM Mg per liter, there is no difficulty in preparing a calibration graph for a given buffer-indicator mixture. Fig. 1 shows a typical graph of this type for one such mixture.

The determination of Mg alone by this method required quantitative removal of Ca. The classical method of precipitation as the oxalate was tested with known standard solutions of concentrations approximating those found in the saliva. A serious complication was the frequently incomplete precipitation of Ca as the oxalate. Recent investigators have encountered this difficulty.[14, 19, 20] With different techniques for precipitation of small concentrations of Ca these authors found 0.015-0.3 mM Ca per liter unprecipitated.

In view of the importance of the oxalate precipitation for the determination of both Ca and Mg in saliva and other biologic fluids, a fairly detailed study was understaken with salt solution concentrations approximating those of the saliva, and using such small volumes as can easily be obtained, in the case of saliva, without stimulation. Mg, or Mg and unprecipitated Ca, was determined by EDTA titration using the technique described in detail at the end of this section, in some of the experiments with slight modifications. The standard solutions used were mixtures of $CaCl_2$ and $MgCl_2$, of fixed concentrations be-

tween 1 and 2 mM Ca and 0.1-0.5 mM Mg per liter. In some tests phosphate was added to the concentration of 5 mM per liter, and HCl to a reaction that prevented precipitation and gave a pH of 4.3 on the subsequent addition of 0.2 volume of saturated ammonium oxalate.

No difference could be detected between precipitations at different pH values between 4 and 7, and no coprecipitation of Mg was ever found. This was in agreement with Holth's observation that magnesium oxalate was never precipitated from solutions of concentrations below 3 mM Mg per liter.[19]

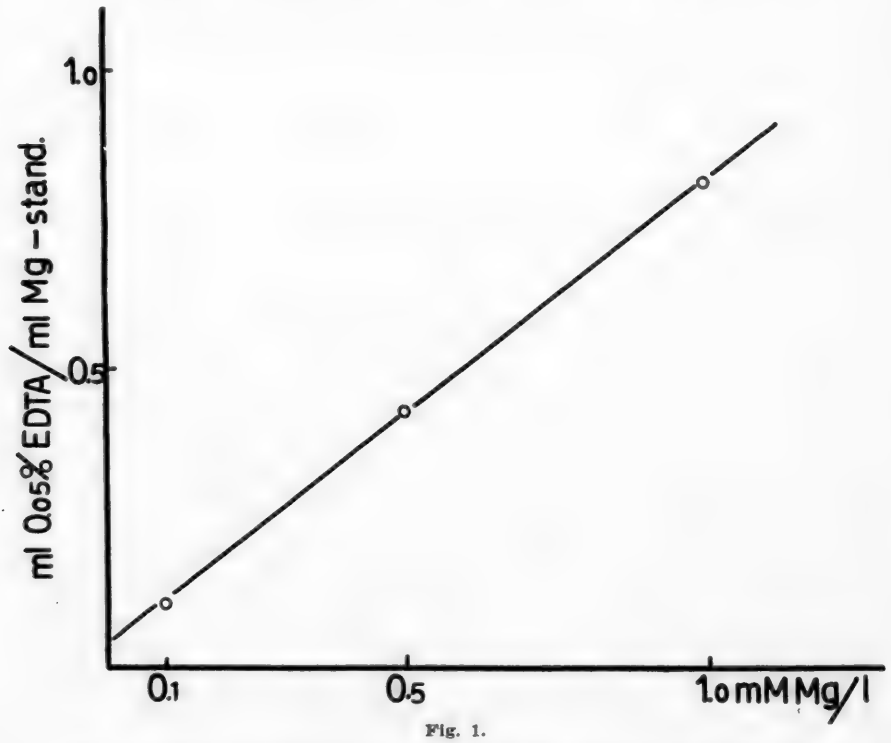

Fig. 1.

The concentrations of unprecipitated Ca were generally of the order, 0.01 to 0.02 mM per liter. No differences were obtained with varying precipitation times exceeding one-half hour. This is apparent from Table I, which shows the results of two typical series of tests in which the titrations were performed by the technique given later. In one of these series, as in some other tests, precipitation was also carried out at 80° C. overnight, followed by several hours' refrigeration; no more Ca was removed. As Table I shows, the different centrifugation periods tested gave no consistently different results; neither did decantation, compared with a suction technique that prevented swirling, or removal of any surface film. In a number of series, these separation techniques were found more reliable than filtration through even the best paper filters or the finest glass filters.

TABLE I

TESTS ON CALCIUM OXALATE PRECIPITATION

PRECIPITATION CONDITIONS	SER. A				SER. 2	
	CENTRIFUGATION 5 MIN.		CENTRIFUGATION 15 MIN.		CENTRIFUGATION 5 MIN.	CENTRIFUGATION 15 MIN.
	DECANT.	SUCTION	DECANT.	SUCTION	DECANT.	DECANT.
30 min., room temp.	0.145	0.156	0.156	0.168	0.162	0.144
3 hours, room temp.	0.160	0.165	0.168	0.165	0.150	0.162
Overnight, room temp.	0.168	0.156	0.156	0.162	0.168	0.168
Overnight, 80° C., + refrig. 4 hours	----	----	----	----	0.168	0.168
	Med. = 0.160				*Med.* = 0.161	

Two milliliters salt solution (1.5 mM Ca, 0.15 mM Mg, 5 mM total phosphate per liter) + 0.4 ml. saturated $(NH_4)_2 C_2O_4$. All centrifugations at about 2,000 r.p.m. Duplicate titrations with 0.05% EDTA solution.

Since the concentration of unprecipitated Ca occasionally reached the order of 0.1 mM per liter, this method could not be regarded as satisfactory, especially for determination of the low Mg concentrations generally occurring in the saliva, 0.15 to 0.25 mM per liter. As a result of this, efforts were made to determine the salivary Mg by the colorimetric titan yellow method.

In the *application of the EDTA titration to saliva,* it seemed essential to ascertain that no disturbances were introduced by its organic constituents or by the Ca complex formation probably occurring in this fluid.[7, 8] Comparative titrations were, therefore, made with saliva both directly and after ashing, the ash being dissolved in 0.1 N HCl to the original volume. It was found that ashing gave consistently higher titers for Ca and Mg, the differences being from 3 to 15 per cent. In addition, spurious end points were frequent in the direct saliva titration. The blue end-point color changed back to violet after some minutes, indicating the liberation of new Ca or Mg ions. Since this might be supposed to take place either from organic or inorganic complexes or from suspended solid particles, it seemed logical to expect an effect from direct acidulation similar to that obtained by ashing with subsequent dissolution of the salt in acid.

Acidulation to the saliva's isoelectric range of pH 2.5 to 3.0 can be effected by adding 0.3 volume 0.1 N HCl. It was found, however, that titration after this treatment gave values between those obtained by the direct titration and ashing procedures. On the other hand, if the common precipitation with trichloroacetic acid was performed by adding 0.3 volume of 10 per cent acid, the recovery was the same as, or somewhat higher than, with ashing. The same results were obtained by precipitation with 0.3 volume normal HCl, which gave about the same pH level (slightly above 1) as trichloroacetic acid. These data are given in Table II.

Control experiments with the addition of trichloroacetic acid in the same proportion to a known salt solution resembling saliva gave no change of the titer. Since, moreover, the agreement between trichloroacetic acid and normal hydrochloric acid treatment was good, these techniques were accepted as giving the most accurate results. No definite explanation can be offered for the lower values found after ashing, but it is known that magnesium ammonium phosphate

in particular is gradually transformed into the pyrophosphate on ignition. In solution the pyrophosphate is changed back to the ortophosphate rather slowly, and it might thus reduce the titer by its strong binding action on Ca and Mg.

Recommended Technique for Determination of Ca and Mg in Saliva.

Solutions (all of reagent grade chemicals in distilled water).—

Ammonia Buffer, pH about 10: 1 vol. M NH_4Cl + 7 vol. M NH_4OH.

Indicator solution: 25 mg. Eriochrome Black T (Eastman Kodak No. P6361) dissolved in 50 ml. ethanol. Stored in refrigerator.

Buffer-indicator mixture: 1 ml. of indicator solution added to each 15 ml. of ammonia buffer. A sufficient volume for titration of the whole analytic series and the necessary Mg standards is made up within a few hours of the titration, because the color is unstable over long periods.

TABLE II

RESULTS OF EDTA TITRATIONS OF SALIVA*

EXP. NO.	ASHING (1)	CCl_3COOH (2)	N HCl (3)	DIFFERENCES (2-1)	(3-1)	(3-2)
1	1.19	1.19		0.00		
2	1.35	1.35		0.00		
3	1.10	1.12		+0.02		
4	0.92	0.82		−0.10		
5	1.24	1.24		0.00		
6	1.34	1.39		+0.05		
7	1.20	1.26		+0.06		
8	0.92	0.99		+0.07		
9	1.18	1.27	1.27	+0.09	+0.09	0.00
10	1.26	1.32	1.32	+0.06	+0.06	0.00
11	1.11	1.18	1.14	+0.07	+0.03	−0.04
12	1.18	1.24	1.20	+0.06	+0.02	−0.04
13	1.15	1.25	1.24	+0.10	+0.09	−0.01
14	1.14	1.22	1.24	+0.08	+0.10	+0.02
15	1.26	1.38	1.33	+0.12	+0.07	−0.05
16	1.22	1.32	1.32	+0.10	+0.10	0.00
17	1.26	1.32	1.32	+0.06	+0.06	0.00
18	1.31	1.25	1.28	−0.06	−0.03	+0.03
Mean differences			No. 1-8	+0.013		
			No. 9-18	+0.068	+0.059	−0.009
			No. 1-18	+0.043		

*After (1) ashing at 650° to 700° C.; ash dissolved in original volume of 0.1 N HCl, (2) protein precipitation with 0.3 vol. 10 per cent CCl_3COOH, and (3) protein precipitation with 0.3 vol. normal HCl. All concentrations are in millimols per liter.

EDTA solution, 0.05 per cent: 250 mg. disodium ethylene diamine tetra-acetate dihydrate dissolved in 500 ml. water and stored in a Pyrex bottle. Stable for months.

Standard solutions of $MgCl_2$: The titer of an approximately 100 mM. $MgCl_2$ solution is determined exactly, e.g., by titration with $AgNO_3$ using dichlorofluorescein as indicator.[22] Strengths of 0.5, 1.0, 1.5, and 2.0 mM per liter are prepared from this stock solution. A drop of toluene is used as preservative.

Procedure.—Mix 1 ml. saliva and 0.3 ml. normal HCl (or 10 per cent trichloroacetic acid) in a centrifuge tube. After one-half hour, centrifuge and decant. Pipette 0.5 ml. volumes in duplo into 12-ml. round-bottomed Pyrex tubes. To each tube add 8 ml. buffer-indicator mixture. Pipette into a similar tube 1.5 ml. distilled water and 8 ml. buffer-indicator mixture. Titrate the

saliva samples with the EDTA solution to a color matching that of the blank tube. Use a burette with immersing capillary point and graduated in 0.01-0.001 ml.; provide a good light source behind the tubes; introduce air bubbling to mix the contents of the titration tube. Ascertain the end-point reading by subsequent overtitration by 0.005 ml.; this will give a more distinctly blue color than the blue of the blank tube. Prepare a linear calibration graph by titrating one standard on each side of the concentration of the saliva samples, using the same buffer-indicator mixture. Read the concentrations of the saliva samples from this graph. Reading times 1.3 = salivary Ca and Mg.

A precision of 1.5 per cent has been obtained with a number of duplicate analyses by this technique.

II. Magnesium Determination by the Titan Yellow Method

If Mg is precipitated as the hydroxide in strongly alkaline solutions of certain dyes—titan yellow, thiazol yellow, brilliant yellow, Clayton yellow—the light yellow color shifts to red. These dyes apparently act as adsorption indicators for $Mg(OH)_2$. Sedimentation of the precipitate can be prevented and the color thus stabilized by the addition of protective colloids, such as starch, gum ghatti, agar, or polyvinyl alcohols.

Early workers who did not use protective colloids had great difficulty with the color instability due to sedimentation.[12, 21] Even with colloids difficulties arose from interfering metals (especially Ca, Al, Fe, and Mn), and phosphate ions. Several authors have tried to neutralize the interference by using compensation solutions containing these ions in high concentrations.[6, 28, 32, 33] A high concentration of polyvinyl alcohol was also used in order to prevent the precipitation which seemed to be the mechanism of these interferences.[34]

In the body fluids interference might be expected primarily from Ca and (PO_4) ions. Previous workers have made conflicting statements on this point, due possibly to their different techniques.[10, 17, 18, 27]

ORIGINAL INVESTIGATION

The titan yellow preparation used in all tests and experiments leading to the technique finally adopted was Eastman Kodak No. P4454. Differences have been reported between the products of various manufacturers.[26] Three polyvinyl alcohols were used: Du Pont's Elvanol 72-51, 51-05, and 70-05. The last grade was preferred since on standing it gave quite a clear solution with no sedimentation.

Solutions of polyvinyl alcohol, titan yellow, and sodium hydroxide were added, in this order, to the samples or standards which had been pretreated as will be described. Different proportions and concentrations were tested, departing chiefly from 3 of the works quoted previously.[10, 17, 27] In conformity with previous reports,[10, 16] it was found that the color development and the agreement with Beer's law were closely dependent on the titan yellow concentration. The variations in color development between different analysis series were found to necessitate the processing of at least 3 known standards along with each series.

Protein precipitation, with 1 volume 10 per cent trichloroacetic acid to 3 volumes of saliva, gave the well-known opalescent centrifugate. On the final addition of the sodium hydroxide solution, the opalescence disappeared. At the same time, however, an increasingly flocculent precipitate appeared which gave rise to rapidly increasing transmittance readings. This was evidently a calcium phosphate precipitate: analysis series with Mg standards in the presence of Ca and inorganic phosphate at about saliva concentrations gave the same precipitates, while these Ca or phosphate concentrations separately had no influence. It seems appropriate, therefore, to judge the influence of these ions on the titan yellow method from their combined effect and not from tests with each ion separately, as has been done in some previous works.

If the calcium of the saliva samples was removed as the oxalate, no precipitation occurred on the addition of sodium hydroxide, and stable transmittance readings were obtained. When the oxalate precipitate was filtered off, it was noticed that most of the proteins were removed at the same time without special measures for their precipitation; only a very slight opalescence remained sometimes, and this disappeared on the final addition of sodium hydroxide. Oxalate precipitation and filtration was, therefore, chosen as pretreatment of saliva for this analysis.

RECOMMENDED TECHNIQUE

Solutions.—

Ammonium oxalate, saturated solution: Add 6 Gm. of reagent grade $(NH_4)_2C_2O_4 \cdot H_2O$ to each 100 ml. of distilled water, heat to near boiling, and allow to cool.

Polyvinyl alcohol, 0.1 per cent: Dissolve 1 Gm. Du Pont Elvanol, grade 70-05, in 1 L. distilled water while heating on water bath and stirring vigorously. Preserve with some drops of toluene.

Titan yellow, 0.01 per cent: Dissolve 50 mg. titan yellow in 500 ml. distilled water. Store in dark bottle in refrigerator.

Sodium hydroxide, 15 per cent: Dissolve 45 Gm. reagent grade NaOH in 500 ml. distilled water.

Procedure.—To 1 ml. saliva, add 0.5 ml. oxalate solution. Mix. Filter after one-half hour. Pipette in order into a Coleman tube, 19 mm. diameter: 1 ml. filtrate, 1 ml. distilled water, 1 ml. polyvinyl alcohol solution, 1 ml. titan yellow solution, and 1 ml. NaOH solution. Mix by shaking between the additions; the water and reagents may be conveniently and accurately added by syringe pipettes.[24] Include blank, 0.1, 0.2, and 0.3 mM Mg standards in each analysis series. Read in spectrophotometer at 520 mμ the wave length of maximum absorption.

Results Obtained With Recommended Technique.—Ten consecutive determinations on a known salt solution containing 0.15 mM Mg per liter, and calcium and phosphate of about salivary concentration gave the mean value 0.163 mM with the standard deviation 0.007 mM. This result, 0.013 mM above the theoretical value, is representative of a greater number of tests carried out with different known solutions. A similar series with saliva gave the values: mean 0.14 mM, standard deviation 0.01 mM.

Ten tests with 0.15 mM Mg. per liter added to saliva gave the mean recovery 0.147 mM, standard deviation 0.021 mM.

No comparison was made with earlier methods for the determination of salivary Mg after precipitation as $MgNH_4PO_4$. It has never been established that such precipitates have the theoretical composition. The limitations of these methods have been clearly recognized by 2 investigators.[4]

Twenty-five analyses on individual samples of stimulated saliva revealed Mg contents varying between 0.13 and 0.23 mM per liter, with the mean value 0.18 mM per liter. Previous authors, using precipitation of $MgNH_4PO_4$, have all given higher average values, for several tested groups about 100 per cent higher, and greater variations.[1, 4, 23] Twenty-nine analyses of resting salivas gave the mean concentration 0.228 mM per liter, standard deviation 0.055 mM per liter.

COMMENT

The incomplete calcium oxalate precipitation at concentrations of the order found in saliva may be regarded as established by the papers quoted and the findings presented in this paper. The complexone titration is also limited by this difficulty as far as the separate determination of Mg is concerned.

The titan yellow method cannot be regarded as ideal but seems to be better than earlier methods. Of these, precipitation as Mg NH_4PO_4 evidently requires a much more elaborate technique for reliability than has generally been used.[4, 22] Although Ca determination by the methods presented here requires 2 different procedures, it is still more convenient than the methods based on analysis of an oxalate precipitate. For clinical estimations on saliva, e.g., in cases of excessive caries or the formation of calculus or salivary duct stones, a single titration of Ca and Mg and deduction of the average Mg content seems quite satisfactory, since the salivary Mg shows rather small variations. For a modified Fosdick test,[8a] the direct complexone titration of salivary Ca and Mg should be almost ideal.

SUMMARY

Two analytic methods for small saliva samples are presented:

1. Determination of the sum of calcium and magnesium by titration with ethylene diamine tetraacetic acid (EDTA), using Eriochrome Black T as indicator.

2. Determination of magnesium by the colorimetric titan yellow method. The analyses revealed lower values for salivary magnesium than have been previously reported.

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MICROBIOLOGIC DIFFERENCES IN THE ORAL CAVITIES OF THE HUNT-HOPPERT CARIES-RESISTANT AND CARIES-SUSCEPTIBLE RATS

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INTRODUCTION

HUNT and Hoppert[1-6] have shown heredity to be a factor in the development of dental caries in rats. By consistent inbreeding and selection, they have produced two strains of rats, a susceptible strain which develops caries within approximately 70 days of age, and a resistant strain which, on the average, develops caries between 400 and 500 days. Studies on the oral microflora of these rats were made by Jay,[7] who recovered lactobacilli more frequently and in greater numbers in the susceptible than in the resistant rats, even in very young animals. The purpose of this study was to investigate, further, differences in the oral microflora of these rats.

EXPERIMENTAL METHODS

A sample of the microflora was obtained by brushing the lower right molars with a sterile cotton swab moistened with a 1 per cent peptone solution. The swab was then placed in 15 ml. of the peptone solution and agitated to disperse the organisms. Dilutions of this suspension were made and plated, using the following media: acidified dextrose agar, acidified potato dextrose agar, Conn's glycerol asparaginate agar, nutritive caseinate agar, tryptone glucose extract agar containing 3 per cent milk, blood agar, nutrient agar, anaerobic agar, eosin-methylene blue agar, lauryl sulfate tryptose broth, mitis salivarius agar, tomato juice agar at pH 5.0, lactobacillus selection medium agar, and also agars adjusted to a pH of 6.5 and 5.0 prepared with the cariogenic diet[8, 9] used in feeding the rats. If, after a thorough trial, a medium did not show significant microbiologic differences between the two strains of rats, its use was discontinued. Swabs made directly from the teeth of rats were streaked on mitis salivarius agar to isolate certain types of streptococci.

Young rats were examined weekly or at more frequent intervals, whereas adult rats were usually selected for single examinations.

RESULTS AND DISCUSSION

Differences in microflora between the caries-susceptible and caries-resistant rats were detected only by highly selective media for lactobacilli and streptococci, namely, tomato juice, LBS, and mitis salivarius agars. The re-

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sults in Table I indicate that lactobacilli occur more frequently in the susceptible rats. In Fig. 1 it may be noted that lactobacilli were not detected until the rats were about 21 days old. There was a slight increase in the frequency of lactobacilli as caries were developing in the susceptible rats, in contrast to a gradual decline in the resistant rats of similar age. This suggests the possibility that the resistant rats are capable of developing a protective mechanism against lactobacilli.

TABLE I

FREQUENCY OF LACTOBACILLI IN CARIES-RESISTANT AND CARIES-SUSCEPTIBLE RATS

MEDIUM	NO. OF EXAMINATIONS		% RATS HAVING 100 OR MORE COLONIES PER MILLILITER OF ORIGINAL SUSPENSION	
	SUSCEPTIBLE	RESISTANT	SUSCEPTIBLE	RESISTANT
Tomato juice agar (pH 5.0)	113	109	89	51
LBS agar	111	109	73	14

In a current study, streak plates on LBS agar have been made directly after swabbing the teeth of rats. About twenty-five rats each of resistant and susceptible strains have been examined weekly or at more frequent intervals starting with 15-day-old rats. Data similar to those reported here have been obtained with the resistant rats, which showed a high percentage of lactobacilli from 25 to 35 days of age. As these rats became older there was a gradual decline in the percentage exhibiting lactobacilli. When the susceptible animals were about 21 days of age, lactobacilli were recovered from all of them. This percentage was maintained before, during, and after the development of caries. Details of this study will be submitted for publication later.

The medium which showed the most striking differences between the resistant and susceptible rats was mitis salivarius agar. *Streptococcus salivarius* and another streptococcus were easily recognized on this medium. *Str. salivarius* appeared as a smooth gum-drop colony; the other streptococcus appeared as a rough, crateriform colony. There was no species described in *Bergey's Manual of Determinative Bacteriology*[10] corresponding to the streptococcus having the rough, crateriform colony. Further studies on the identification of this organism are being made to determine if it is a variant of *Str. salivarius*. The frequencies at which both organisms were recovered from the two strains of rats may be noted in Table II. *Str. salivarius* was found in both susceptible and resistant animals but was recovered twice as frequently in the susceptibles. The streptococcus producing the rough, crateriform colony was found in every resistant rat and in only 18 per cent of the susceptibles. These susceptibles consisted of twenty-four animals, twenty-two of which came from two families In the succeeding generation this streptococcus was recovered from 83 per cent of the progeny of these rats, but was not detected in other susceptibles. It continued to appear in nearly all the resistant animals. This suggests that heredity may have an important influence on the oral microflora.

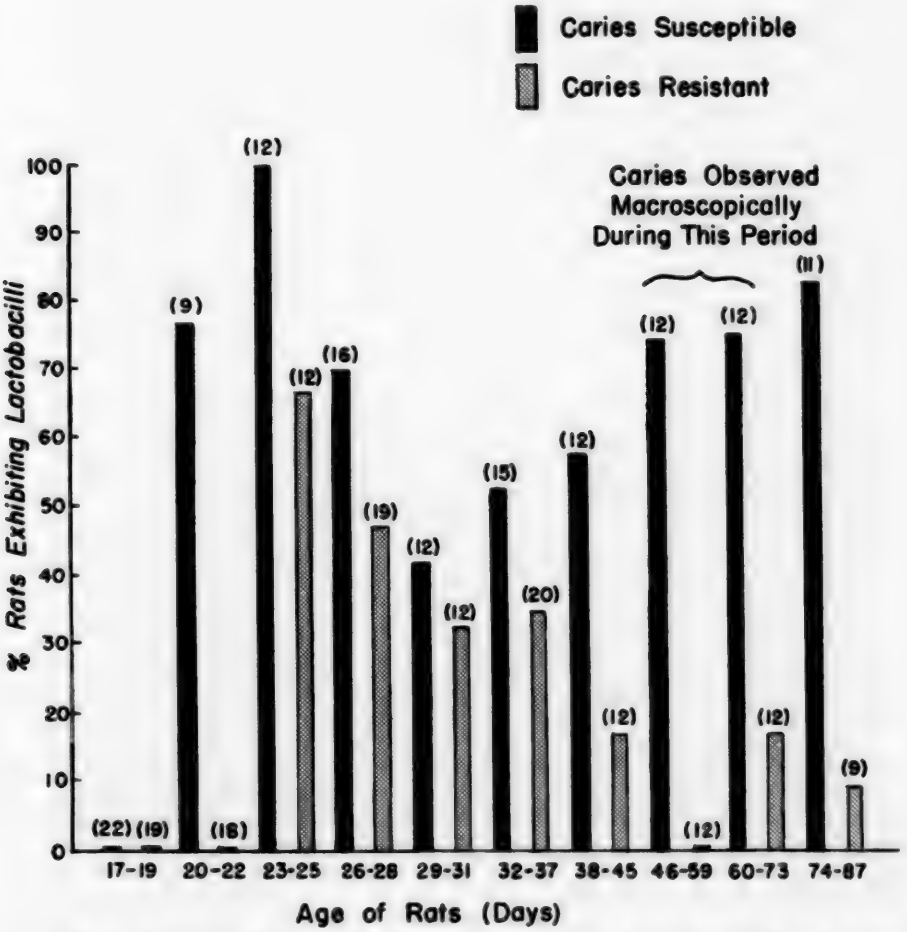

Fig. 1.—Frequency of lactobacilli in the oral cavities of caries-susceptible and caries-resistant rats at various ages using LBS agar. (Numbers in parenthesis indicate number of animals examined.)

TABLE II

FREQUENCY OF THE TWO STREPTOCOCCI FROM CARIES-RESISTANT AND CARIES-SUSCEPTIBLE RATS

ORGANISM	NO. OF EXAMINATIONS	SUSCEPTIBLE (%)	RESISTANT (%)
Str. salivarius	134	96	47
Rough, crateriform streptococcus	134	18	100

The appearance of this streptococcus in all the resistant animals indicated that the organism might be a factor in the delay or prevention of caries. Experiments were carried out to determine whether this organism might have acid-neutralizing or antibiotic activity. It apparently did not have any acid-neutralizing activity, as it was found to be as acidogenic as *Str. salivarius* when grown in a broth prepared from the diet used in feeding the rats. It did exhibit slight antibiotic activity against lactobacilli and *Str. salivarius.* Whenever this organism was found in susceptible rats, it occurred in as large numbers as in resistant rats, and caries developed in the usual time. It seems unlikely, therefore, that this organism delays or prevents caries in the resistant rats.

SUMMARY

The oral microflora of caries-resistant and caries-susceptible rats was compared on many types of bacteriologic media. Differences in microflora between the two strains of rats were detected only by highly selective media for lactobacilli and streptococci, namely, tomato juice, LBS, and mitis salivarius agar.

Lactobacilli and *Str. salivarius* appeared more frequently in the caries-susceptible rats.

Lactobacilli were not detected until the rats were about 21 days old. The frequencies with which these organisms were recovered tended to increase as the susceptibles developed caries, in contrast to a decline in the resistant animals.

An unidentified streptococcus, which appeared as a rough, crateriform colony on mitis salivarius agar, was found in all the resistant rats which were examined and in 18 per cent of the susceptibles. The progeny of these susceptibles also exhibited this organism, which suggested that heredity may influence the types of microorganisms present in the mouths of rats.

The authors wish to thank Miss Lisa Neu, Mr. Irving Dahljelm, Dr. H. J. Stafseth, Dr. F. W. Fabian, and Dr. R. N. Costilow of the Department of Bacteriology and Public Health of Michigan State College for their valuable cooperation in this project.

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THE ACTION OF ORAL BACTERIA ON COLLAGEN

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ALTHOUGH it is generally agreed that the role of the oral microorganisms in the pathogenesis of chronic periodontitis is one of considerable importance, many aspects of the problem are still obscure, such as the precise point of attack and the possibility of migration of organisms in the periodontal tissues, the method of bacterial action, and the effect of predisposing factors. The investigation reported here was undertaken to shed some light on one of these problems, that of the nature of bacterial action.

MATERIALS AND METHODS

Azocoll.—Commercial hide powder, which is a rich source of collagen, was used as the substrate, the powder being coupled with an azo dye to give the bright reddish purple substance "azocoll," according to the method of Oakley, Warrack, and van Heyningen.[8] These workers used azocoll as an indicator for the κ toxin (collagenase) of *Clostridium welchii*, which breaks down the collagen, thus liberating the dye.

In the experiments reported here, a series of sterile screw-capped bottles was prepared, each containing a small amount of azocoll and 5 ml. of serum broth. In order to avoid contamination, the azocoll was added in the form of a suspension in acetone (0.04 Gm. in 5 ml.), 10 drops of the suspension being placed in each bottle, the acetone evaporated, and the broth then added immediately prior to use.

Material from eighteen persons was examined, in twelve of whom a clinical diagnosis of periodontitis simplex had been made. These patients showed pocket depths varying between 5 and 10 mm., with accompanying loss of alveolar bone. Subgingival calculus was present in all cases. Specimens were collected by means of sterile paper points inserted to the depths of the pockets, after drying the surrounding teeth with sterile cotton and packing off the selected area. The remaining six subjects were persons whose gingivae appeared clinically healthy, showing normal stippling, sulci no deeper than 1.5 mm., and no evidence of calculus. Specimens were similarly collected, by introducing paper points into the sulci.

Four paper points were taken from each subject, two points being placed in bottles each containing azocoll and 5 ml. of broth, and the remaining two in bottles each containing 50 ml. of serum broth. These media were then incubated aerobically and anaerobically for forty-eight hours. At the end

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of this period the azocoll bottles were examined for evidence of disintegration of the collagen, and subcultures were made on horse blood agar plates. The resulting colonies were separated and identified, and the strains of organisms thus obtained were each transferred to further bottles of azocoll and broth and incubated for forty-eight hours.

From the 50 ml. quantities of broth, Seitz filtrates were prepared and tested for their action on azocoll.

RESULTS

Although slight alterations of the azocoll were detectable at the end of twenty-four hours' incubation, it was not until forty-eight hours that definite readings could be made. Incubation for longer periods did not materially alter the readings. The degree of disintegration of the azocoll varied from case to case, a positive result being taken as one in which actual breakdown or disintegration of the collagen was observed. A negative result covered those cases in which there was no change in the azocoll or at the most a slight discoloration.

Table I shows the results obtained using the primary cultures and culture filtrates. No appreciable difference between aerobic and anaerobic culture was observed.

TABLE I

EFFECT ON AZOCOLL OF CULTURES AND CULTURE FILTRATES FROM NORMAL GINGIVAL SULCI AND PERIODONTAL POCKETS

	NUMBER	DISINTEGRATION OF AZOCOLL	
		CULTURES	CULTURE FILTRATES
Healthy subjects	6	6	0
Cases of periodontitis	12	12	1

Table II shows the results obtained with the separate strains of organisms obtained from the primary cultures.

DISCUSSION

Until comparatively recently, the actual mode of bacterial action on the periodontal tissues was a matter for conjecture, but now a number of investigations have led to some clarification of the problem. Earlier workers[2,3] demonstrated the presence of microorganisms in fluid aspirated from the gingivae. Later investigations cast doubts on the view that organisms were to any extent present within the tissues; tissue destruction was considered to be due rather to permeation by toxins derived from bacteria in the debris of the periodontal pocket. Such toxins, however, had never been isolated, although within recent years it has been shown that certain enzymes capable of causing changes in the tissues are present in saliva, or may be elaborated by oral bacteria. Lisanti[6] and Mahler and Lisanti[7] have shown that hyaluronidase, which acts on the intercellular cementing substance, is present in the saliva both in healthy persons and in those suffering from periodontal disease, especially the latter, and Aisenberg and Aisenberg[1] have produced appearances in the gin-

TABLE II

STRAINS OF ORGANISMS ISOLATED FROM GINGIVAL SULCI AND PERIODONTAL POCKETS OF EIGHTEEN PERSONS. FOR EACH CASE THE NUMBER OF STRAINS DISINTEGRATING AZOCOLL IS GIVEN IN COLUMN a AND THE NUMBER WITHOUT EFFECT ON AZOCOLL IN COLUMN b

ORGANISMS	HEALTHY PERSONS												CASES OF PERIODONTITIS																								TOTALS
	1		2		3		4		5		6		1		2		3		4		5		6		7		8		9		10		11		12		
	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	
Streptococcus viridans	1	1	2		2	1	1	2	2	1	1	1		2	2	1	1	1		2	2	2	1	1	1		1	1		3	2	2	1	1		1	43
Streptococcus β-hemolyticus																							1		1												2
Nonhemolytic streptococci					1						1		1		2				1		1		2	2	1	1	1	1	1	1		1	2	1	1		22
Pneumococcus															1																						1
Staphylococcus	1																		1				1		1						1						5
Micrococcus catarrhalis		1							1																1						1	1			1	1	7
Diphtheroid bacilli											1		1				1		1	1	1		1		1			1	1				2		1		13
Subtiloid bacilli			1				1		1				2								1					1	1								1		9
Friedländer's bacillus																	1												1		2	1		1		6
Micrococcus tetragenus					1																																1
Totals	2	2	3	0	4	1	2	2	4	2	2	1	4	2	2	4	2	2	2	3	4	4	4	5	3	4	3	4	2	5	4	6	4	4	5	2	109

Total number of strains isolated from eighteen persons = 109.
Number of strains disintegrating azocoll = 56.
Number of strains without effect on azocoll = 53.

givae of monkeys comparable in some respects to those observed in human disease, by the injection of hyaluronidase. Sreebny and Engel[11] have shown that a fraction of saliva is capable of depolymerizing azocoll, and Roth and Myers[10] have demonstrated the disintegration of collagen paper in cultures from periodontal pockets.

The experiments reported here show that many strains of the common oral microorganisms, recovered both from healthy mouths and from the pockets in cases of periodontal disease, are capable of disintegrating collagen, although no attempt was made to cultivate the more delicate organisms or to evaluate the effect of those organisms that cannot be cultured. It is of interest to note that in only one case could a collagenase-like effect be obtained with a bacterium-free culture filtrate; in all the remaining cases the effect on collagen was produced by actual cultures. This need not be taken as indicating that the bacteria concerned produce no separable enzymic products, however, since the concentrations may be so small in most cases that the bacterial cells must themselves be present to produce effects detectable under the conditions of the experiment. Furthermore, chronic periodontitis is of slow natural evolution, so that any toxic or other bacterial products concerned in the pathogenesis of the condition must necessarily be liberated in very small amounts, over a long period.

Although many of the readily isolated oral organisms are thus capable of disintegrating collagen, the role of others of the bacterial flora must be taken into account. Thus Hemmens and Harrison[5] have recovered, from cases of periodontitis, such organisms as *Micrococcus gazogenes, Fusiformis nucleatus* and other fusiforms, *Bacterium melaninogenicum,* and leptotrichia in appreciable proportions, and Rosebury[9] has found spirochetes, fusiform bacilli, and vibrios to be in evidence in pathologic conditions of the gingivae. These authors have also shown that organisms of the types just mentioned can produce abscess formation on injection into guinea pigs. Box[4] has also paid attention to the role of specific organisms, and has claimed that *Leptothrix falciformis* is of etiological significance in the production of foci of necrosis.

There is thus a growing body of evidence to show that many of the organisms of the oral cavity must be considered potential pathogens, so far as the periodontal tissues are concerned, and although most bacteriologic studies made in recent years have tended to emphasize the role of particular groups of organisms, the part played by bacteria generally regarded as purely commensal should not be forgotten.

SUMMARY

Cultures were made from the pockets of twelve persons with periodontal disease and from the gingival sulci of six healthy persons. One hundred nine strains of organisms were isolated, fifty-six of these being capable of causing the disintegration of collagen in vitro.

It is concluded, from this and other evidence, that many of the organisms of the oral cavity are potential pathogens, and that the role of organisms generally regarded as purely commensal should be borne in mind.

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THE LOCALIZATION OF POLYSACCHARIDES IN DEVELOPING TEETH

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INTRODUCTION

THIS investigation deals with the localization and interrelationship of mucopolysaccharides and acid polysaccharides in the developing tooth. Carbohydrate complexes have been recognized in calcifying structures for some time, and various roles have been assigned to them in an attempt to explain the mechanism of calcification. We shall describe the localization of muco- and acid polysaccharides in the various components of the developing tooth and correlate their appearance in terms of chronology and localization with the calcification of dentin and enamel.

Holmgren[8] described the occurrence of metachromasia in the ground substance of the embryonic pulp and of the stellate reticulum. This observation was confirmed by Wislocki, Singer, and Waldo,[15] who also observed metachromasia in some components of the adult tooth. Mucopolysaccharides identified by the periodic acid-Schiff reaction were reported by Engel[3] to occur in the outer enamel epithelium, the stellate reticulum, the stratum intermedium, and in ameloblasts during the time calcification of enamel takes place. This author also identified mucopolysaccharides in the membrana preformativa, in functional odontoblasts, and in the cells of the pulp. In a subsequent paper, Wislocki and Sognnaes,[16] dealing with the same topic, stated that the processes of the odontoblasts, the matrix of dentin, and the reticulum of the dental pulp are periodic acid-Schiff positive; the enamel and stellate reticulum are faintly positive or negative.

MATERIAL AND METHODS

The material used in these studies consisted of the lower incisor teeth of developing pig embryos ranging in size from 130 to 170 mm. Although several methods of fixation were utilized, Carnoy's was routinely used for material to be stained with the periodic acid-Schiff procedure, and Helly's was found to be most satisfactory for subsequent treatment with metachromatic dyes. The mucopolysaccharides were identified according to the method of Hotchkiss.[9] Both toluidine blue and methylene blue were used to identify metachromatic constituents of the tooth. These dyes were buffered with McIlvaine's buffer at a pH ranging from 2.0 to 8.0 at 0.05 per cent concentration of dyestuff. Most satisfactory results were obtained at a pH ranging from 6.4 to 6.6. The preparations were observed in the wet condition according to the directions of

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Hempelmann[7] to observe the maximum metachromasia present in tissues before running the sections through the higher alcohols. Sections digested in hyaluronidase (Wyeth)* were subjected to concentrations of the enzyme equivalent to 100 TRU per cubic centimeter. Digestion was carried out at approximately 37° C. for ten to twelve hours at a pH of 6.0 to 6.2.

METACHROMASIA

Examination of the components of the enamel organ reveals that the ground substance of the stellate reticulum, especially well preserved in sectioned material following Helly's fixative, is intensely metachromatic. The ground substance in these preparations consists of a mass of fine granules which lie embedded in a coagulum. The cells of the stellate reticulum and of the stratum intermedium, however, stain orthochromatically. The ameloblasts also stain blue, but Tomes's enamel processes are weakly metachromatic. While pulp contains several histologic components, metachromasia occurs in but one constituent, the ground substance.

Special attention has been directed to the study of the development of dentin with reference to the metachromatic properties this tissue exhibits. For purposes of description, its development is arbitrarily divided into three stages: (1) the formation of predentin, (2) the initiation of calcification, and (3) the formation of a uniformly calcified matrix.

Predentin.—The formation of predentin consists in part of the establishment of the reticular basement membrane between the ameloblasts and the odontoblasts and the subsequent orientation of its fibers in a ground substance. These fibers or tufts are arranged along the future dentinoenamel junction and subsequently become incorporated in the calcifying matrix. By the time these (Korff's) fibers appear, the developing odontoblasts elaborate a protoplasmic process which comes to occupy a radial position in the newly developing tissue. Predentin consists, then, according to our observations, of reticular fibers, odontoblastic processes, and a semifluid ground substance which intimately surrounds the other elements. We observe that the ground substance is intensely metachromatic; odontoblastic fibrils are weakly metachromatic, while Korff's fibers are intensely orthochromatic.

Initiation of Calcification.—This stage is recognized by the appearance of small globular concretions which first become discernible by the von Kossa as well as other staining procedures. Those areas usually referred to as calcoglobules are believed to consist of aggregations of mineral crystals. The earliest observed calcoglobules stain metachromatically; as these structures increase in size and become more numerous they gradually lose their metachromatic coloration, first in the center and subsequently in the periphery, and stain blue (orthochromatically). Orthochromasia is likewise the characteristic color reaction of newly calcified dentin.

Uniformly Calcified Dentin.—This dentin located in the zone adjacent to the dentinoenamel junction in the incisor of 170 mm. embryo is calcified uni-

*We are indebted to Wyeth Incorporated for supplying the hyaluronidase used in these studies.

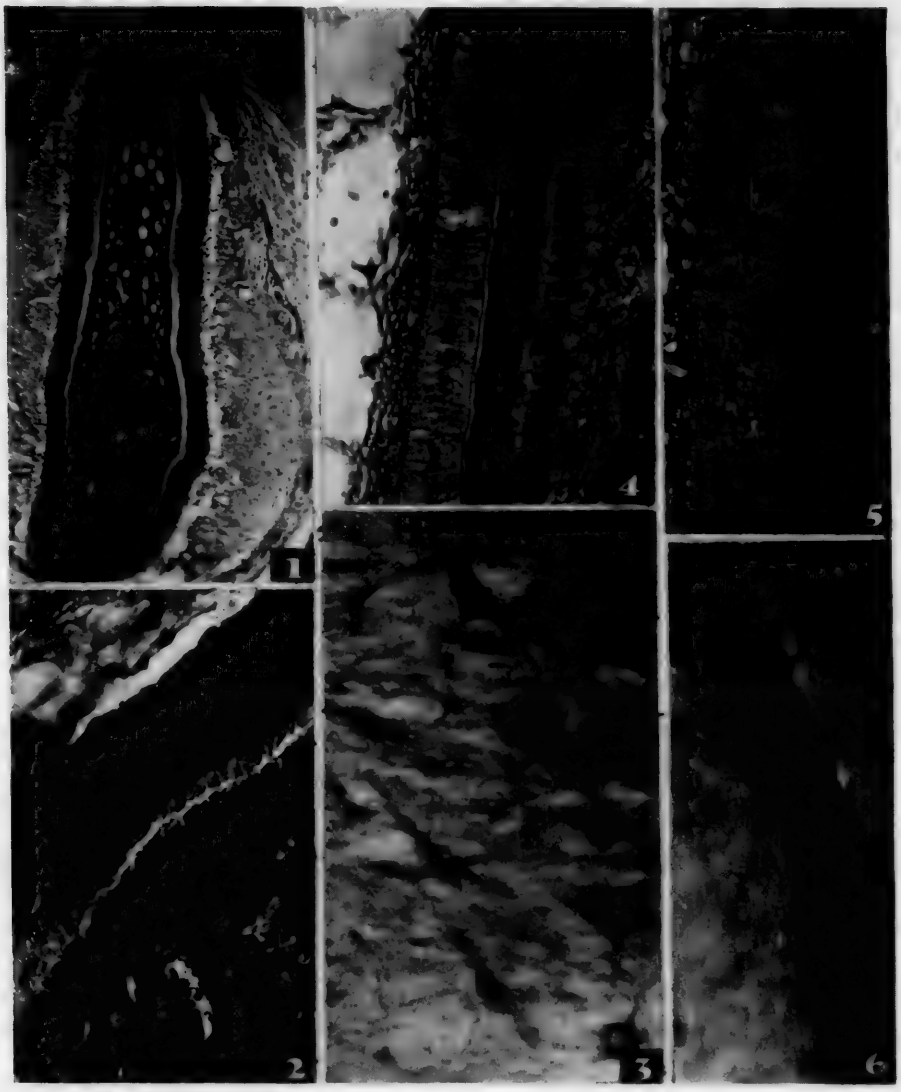


Fig. 1.—Low-power photomicrograph of sagittal section of incisor of 130 mm. specimen to show stage of development of calcified tissues. Stained with toluidine blue.

Fig. 2.—Higher magnification of strip of calcifying tooth. Predentin, the light area stains metachromatically, the more peripheral dark-staining dentin stains orthochromatically. Toluidine blue, 130 mm.

Fig. 3.—High-power photomicrograph of stellate reticulum showing ground substance which stains metachromatically and cells which stain blue. Toluidine blue stain, 130 mm.

Fig. 4.—Strip of calcifying tooth stained with periodic acid-Schiff stain. Amelobosts and odontoblasts and predentin stain at about the same intensity. Calcified dentin, more intensely stained at 130 mm. specimen.

Fig. 5.—High-power photomicrograph of root portion of tooth of 130 mm. specimen showing intense stain of basement membrane following periodic acid-Schiff procedure.

Fig. 6.—High-power photomicrograph showing odontoblasts and Tomes's processes which were stained by periodic acid-Schiff procedure.

formly and apparently to a greater degree than the zone adjacent to predentin. Unlike the two previous stages of development just described, this peripheral zone of calcified dentin stains neither metachromatically nor orthochromatically, but is relatively colorless. In the orthochromatically stained dentinal matrix, Tomes's dentinal fibrils lose their faint metachromatic color and become orthochromatic. The fibers of the matrix retain their blue coloration, as was the case in predentin. In the more highly calcified areas of dentin, neither Tomes's fibers, the collagen fibers, nor the ground substance exhibit any appreciable tinctorial property.

Although there is no reticular fibrous component involved in the formation of enamel, there is, nevertheless, a membrane elaborated which occupies a position on the dentinal aspect of enamel. This membrane assumes a green coloration. As enamel forms, the ameloblasts are blue, and Tomes's enamel processes appear faintly metachromatic. The earliest increments of enamel laid down stain metachromatically. As calcification of enamel proceeds, the matrix and Tomes's processes first assume a blue coloration, while in the older increments of enamel, tinctorial properties disappear entirely.

MUCOPOLYSACCHARIDES

Examination of the developing tooth treated by the Hotchkiss method to show the localization of mucopolysaccharides exhibits the following: The components of the enamel organ reveal positive staining reactions in both the cellular components and the ground substance of the stellate reticulum. In addition, numerous extracellular glycogen granules are observed in this tissue. The cells comprising the stratum intermedium show a gradual acquisition of a Hotchkiss-positive staining reaction in the region of the mid-portion of the developing tooth, whereas the cells in the root and crown portion are relatively negative.

Ameloblasts.—In the young developing ameloblasts, the staining reaction is relatively absent. As the cells differentiate and undergo elongation, the intensity of the stain gradually increases; this heightened staining intensity is maintained throughout the period that enamel is elaborated. When the ameloblast is fully mature and associated with enamel production, mucopolysaccharides are visible in both the infra- and supranuclear portions of the cell. The most intense staining reaction in these cells occurs in the numerous filamentous structures occurring in the cytoplasm. These structures also stain intensely with methylene blue and pyronine, Johnson and Bevelander.[10] Tomes's enamel processes and embryonic enamel are also faintly stained.

Pulp.—The constituents of the developing pulp, namely cells, fibers, and ground substance, are Hotchkiss positive. The basement membrane and Korff's fibers are also intensely stained. When predentin is laid down, the differentiating odontoblasts and the predentin matrix gradually assume a pink coloration. A calcification of dentin proceeds, the calcified parts of the dentin and the odontoblasts become intensely colored.

Hyaluronidase-Treated Material.—Sections digested with hyaluronidase and subsequently stained by the Hotchkiss method reveal a partial removal of

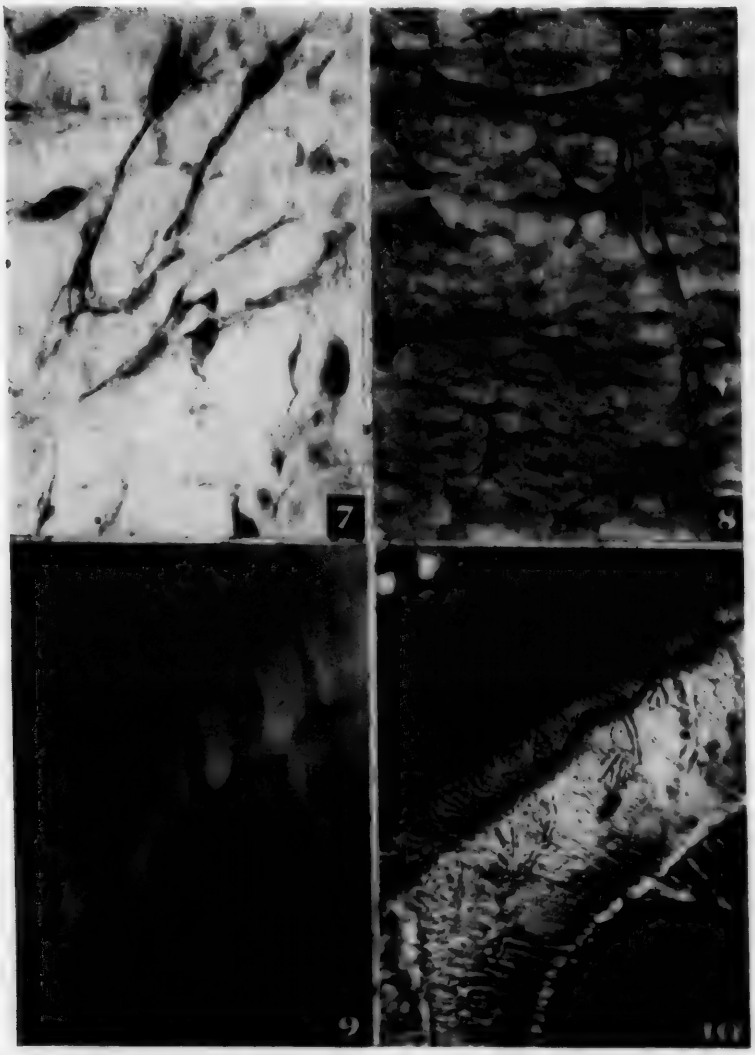


Fig. 7.—Stellate reticulum digested with hyaluronidase and subsequently stained with toluidine blue. Note complete removal of ground substance. Compare with Fig. 3.

Fig. 8.—Stellate reticulum stained by the periodic acid-Schiff procedure. Ground substance and cells moderately positive.

Fig. 9.—Odontoblasts, odontoblastic processes, and dentin photographed under oil immersion. Stained with toluidine blue. The odontoblasts and the walls of the dentinal tubules are basophilic (blue), predentin and contents of tubules metachromatic, the peripheral dentin and Korff's fibers orthochromatic.

Fig. 10.—Coronal portion of incisor of a 150 mm. specimen. Photograph shows dark blue-staining ameloblasts, light zone of newly formed enamel (metachromatic), a calcified zone of enamel which appears dark and stains orthochromatically. The predentin appears very light (metachromatic), the adjacent calcifying zone dark (orthochromatic), and the most peripheral uniformly calcified area also appears light because of lack of any chromatic reaction on the part of the toluidine blue reaction.

the ground substance in the pulp. This partial removal of the ground substance following digestion appears to make the basement membrane and Korff's fibers more clearly delineated. When sections of the developing tooth were digested with hyaluronidase and then stained, the metachromatic property of the ground substance of the stellate reticulum was completely lost, while the pulp, dentin, and enamel were not noticeably affected by this treatment.

DISCUSSION

We have described, in so far as methods permit, the appearance and distribution of polysaccharides in the developing, calcifying tooth. Our observations show that mucopolysaccharides have a wide distribution in the developing tooth. They occur in all the components of the stellate reticulum and the dental pulp. In regard to dentin and enamel, it is interesting to note that Hotchkiss-positive staining material is widely dispersed in the matrices of these tissues, and further, with the advent and increase in calcification, the intensity of the staining reaction is considerably enhanced. The increased intensity of the mucopolysaccharide reaction (content) of calcifying dentin and enamel is similar to previous observations reported for bone, Bevelander and Johnson,[1] Heller-Steinberg,[6] Cobb,[2] Pritchard,[14] and others.

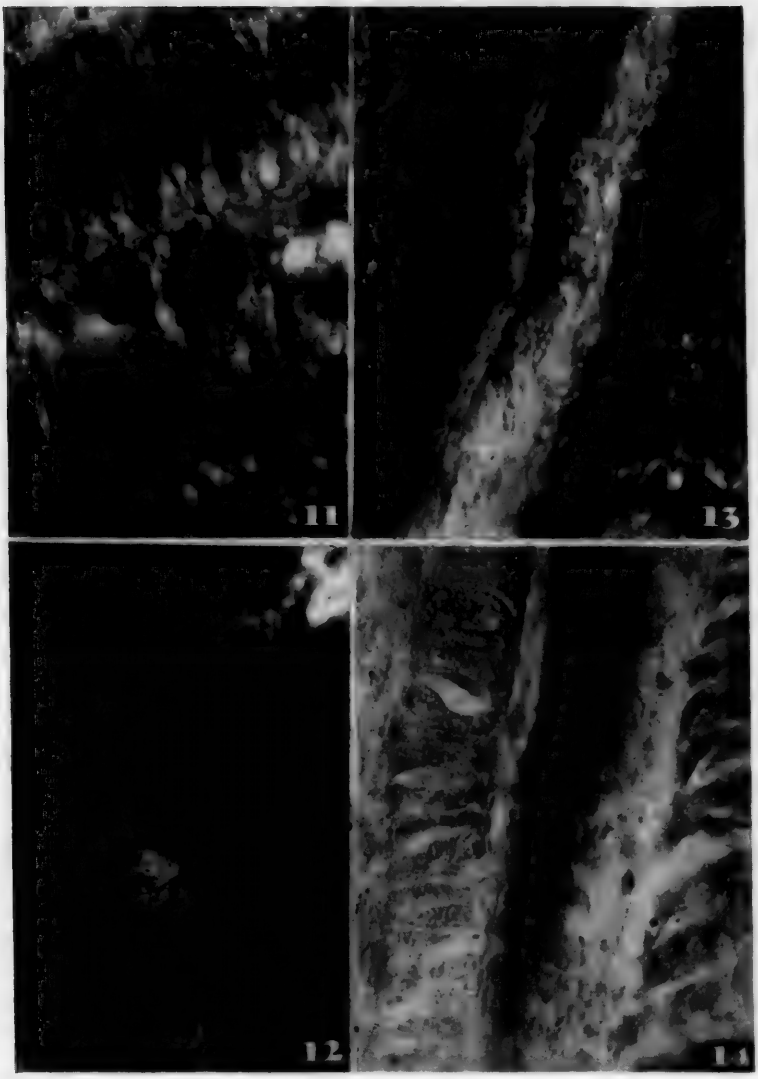
Another correlation which stems from our observations concerns the gradual appearance of mucopolysaccharides in the odontoblasts and ameloblasts. In a previous report (Johnson and Bevelander[10]), a correlation between the presence of alkaline phosphatase and ribonucleic acid was established in relation to the functional state of these cells and calcification. In this connection, Moog and Werner,[13] in a study of the occurrence of neutral polysaccharides at the sites of high phosphatase activity, suggested that nonacidic mucopolysaccharides serve as a cytoskeletal structure which provides a directive orientation in an environment suitable for the activity of phosphatase.

The studies of Gersh and Catchpole[4] also emphasize the possible enzymatic role of mucopolysaccharides in tissues, and Cobb[2] has suggested that the depolymerization of this carbohydrate complex may be associated with an increase in reactive groups which combine with calcium.

Metachromasia, indicative of acid polysaccharides, is confined to extracellular sites in the developing dental tissues.* The acid polysaccharide which occurs in the ground substance of the stellate reticulum appears to be incorporated in the enamel matrix. Similarly, the metachromatic ground substance of the dental pulp, we believe, is the source of this substance which occurs in dentin.

The loss of metachromasia in the ground substance of the stellate reticulum following digestion with testicular hyaluronidase suggests that the polysaccharide which is depolymerized by this treatment in chondroitin sulfate A, Meyer.[12] The failure of hyaluronidase digestion to alter appreciably the metachromatic properties of dentin and the pulp indicates that the acid poly-

*With the possible exception of nuclear metachromasia, which was not carefully examined in this study.



Figs. 11 and 12.—Two different areas of 130 mm. specimen stained with toluidine blue to show Korff's fibers staining orthochromatically while the ground substance stains metachromatically.

Fig. 13.—High-power photomicrograph showing initiation of calcification of dentin. Stain, toluidine blue. Note uncalcified dentin light (metachromatic) calcoglobules and calcified areas dark (orthochromatic).

Fig. 14.—Strip similar to that shown in Fig. 13, stained by the von Kossa method to show areas of calcification.

saccharide occurring in these tissues differs from that found in the stellate reticulum. Since Hess and Lee,[5] have isolated chondroitin sulfate from dentin, it seems probable that the substance we identify in this tissue is chondroitin sulfate B or C.

Although the identification of substances in tissues by chemical methods cannot be duplicated in all respects by histochemical methods, the two technics frequently complement each other in many instances. Moreover, histochemical studies may elucidate information not readily obtained by the usual methods of chemical identification. An example of this latter situation, we believe, is brought out in the interpretation of the reaction of developing dentin when treated with dye such as methylene or toluidine blue.

The observed histochemical reaction of developing dentin shows three different chromatic states following treatment with methylene blue: predentin (uncalcified) dentin stains metachromatically, initially calcified dentin stains orthochromatically, while the more highly calcified portions do not stain appreciably at all. Aside from the observed color reactions which result following treatment with metachromatic dyes and the correlation existing between these differences, and the degree of calcification, this phenomenon appears to be puzzling only in the latter stage, inasmuch as chondroitin sulfate appears to be present in dentin. The experiments of Levine and Schubert,[11] however, have shown that an increase in calcium ions in combination with chondroitin sulfate reduces or abolishes metachromasia. Therefore, it appears that an increase of calcium ions in the calcifying matrices of bone and dentin might likewise account for a loss in the metachromasia of these tissues. The increase in metachromatic staining in the matrices of calcified bone and dentin following demineralization lends support to this concept.

In view of the many facts which must be assembled and tested before a relatively clear picture of the mechanism of calcification becomes available, we cannot presume to assign further the possible physiologic role which polysaccharides play in the development of the tooth or the process of calcification. It appears evident, however, that as more histochemical studies of calcifying tissues are completed, the mechanisms involved in the elaboration of calcifying tissues must be re-evaluated.

SUMMARY

A histochemical study of the developing tooth has been made with special reference to the localization of polysaccharides in the several components.

Mucopolysaccharide is widely distributed in the several components of the tooth; of especial interest is its gradual increase in intensity in functional ameloblasts and odontoblasts. With the onset of calcification, dentin likewise shows a corresponding increase in mucopolysaccharide content.

Metachromasia, presumably indicative of acid polysaccharides, is confined almost exclusively to extracellular regions. Intense metachromasia was observed in the ground substance of the dental pulp and of the stellate reticulum of the enamel organ. As enamel and dentin are elaborated, the ground substances of these two tissues exhibit metachromasia. With the advent of calci-

fication, the initially calcified increments stain orthochromatically (blue). Older, more highly calcified enamel and dentin do not stain with either toluidine or methylene blue. The probable failure of calcified tissues containing acid polysaccharides to stain metachromatically has already been referred to.

Sections of the developing tooth digested with testicular hyaluronidase subsequently show a loss in metachromasia of the ground substance of the stellate reticulum. Failure of this enzyme to alter appreciably the metachromasia of the other components of the tooth indicates that the acid polysaccharide complex of the enamel organ differs from that which is associated with the mesodermal derivatives such as the pulp and dentin.

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HISTOCHEMICAL REACTIONS OF THE LAMPREY MOUTH

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FROM a morphologic standpoint, the mouth and teeth have occupied a prominent place in research on evolution. Gregory[8] has made particularly fascinating observations on evolutionary patterns which have either perished or persisted from the first toothlike organs of the lamprey to the complex oral structures of man. Recent progress in the use of histochemical technics offers intriguing possibilities for extending and correlating some of the morphologic and chemical aspects of evolution. Following previous observations on the histochemistry of the mouth of man and other higher mammals,[16] an initial attempt has been made to explore the presence and location of similar histochemical reactions of the oral cavity and the horny "teeth" of the lamprey.

MATERIAL AND METHODS

The material used for this study was obtained from the Exeter River in New Hampshire. Appropriate fixatives for a variety of histochemical methods were brought along to the river bank, some of the fixatives stored in ice and others mixed in required proportions at the location. Freshly killed animals were dissected on the spot and samples of the oral tissues placed directly in fixatives. The specimens were next brought back to the laboratory of this school and processed for the following studies:

Morphology.—Several specimens were examined grossly, dissected, and prepared for histologic examination with routine stains.

Reactions for Keratin, Sulfhydryl, and Disulfide Groups.—For this purpose, we have used Ebbinghaus' orange II method[6] for keratin and the method of Chevremont and Frederic,[2] which reveals sulfhydryl (SH) groups. Masson's triacid stain served as an auxiliary means of comparing cornified and noncornified structures.

In addition to these methods, we have been able to add two new procedures for SH groups and disulfide bonds recently developed by Barnett and Seligman.[1] One reaction is for demonstration of free SH groups, the other for SH and disulfide bonds combined. The presence of free SH groups is revealed by a reddish to blue color reaction, depending upon the concentration of SH groups.

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Reactions for Mucopolysaccharides and Glycogen.—Metachromatic staining was investigated in specimens fixed in 4 per cent aqueous solution of basic lead acetate for twelve to twenty-four hours, according to the method of Holmgren.[9] This was followed by postfixation in 10 per cent neutral formalin for twelve to twenty-four hours, as recommended by Sylven.[15] Deparaffinized sections were stained in a 0.5 per cent solution of toluidine blue in 5 per cent alcohol for thirty minutes and examined for the presence of metachromatic substance, using a yellow source of light.

Basophilic staining was investigated by a method designed for the characterization of acid proteins, especially acid mucopolysaccharide and nucleoprotein.[5, 13, 14] For this purpose, the tissues were fixed in Zenker's fluid for twelve to twenty-four hours. Paraffin sections were stained in a buffered solution of methylene blue. Acidic substances continue to stain at the lower pH range by this procedure, and this basophilic reaction, coupled with the demonstration of metachromasia after staining with toluidine blue, is considered indicative of the presence of an acid mucopolysaccharide.[5]

The periodic acid-Schiff method of McManus[12] and Hotchkiss[10] was utilized to investigate glycogen and other carbohydrates. This reaction depends upon the oxidation of carbohydrates by periodic acid, with the formation of aldehydes which recolorize Schiff's leukofucshin reagent.[10, 11] Glycogen can be distinguished from other positive-staining substances by exposing control sections to salivary amylase before staining. Besides glycogen, this method stains some mucopolysaccharides (for example, epithelial mucus) and fibrin, besides other substances of unknown nature. For this method, the specimens were fixed in Rossman's fluid (90 c.c. of absolute alcohol saturated with picric acid and 10 c.c. of 40 per cent formaldehyde) for twelve to twenty-four hours at 1° C.

Alkaline phosphatase was demonstrated by the method of Gomori.[7] After fixation in chilled 80 per cent alcohol, specimens were prepared as paraffin sections and incubated in glycerol phosphate for three hours at pH 9.4. Sections, omitting the substrate, were similarly prepared for control.

Lipids were demonstrated in tissues fixed in 10 per cent neutral formalin and prepared as frozen sections. The sections were stained in a 70 per cent solution of alcohol saturated with sudan IV and in sudan black B, respectively, for thirty minutes, after which they were rinsed for a moment in 70 per cent alcohol, washed in water, and mounted in glycerin jelly.

Polarized light: As an auxiliary method, sections were examined in polarized light in order to determine the location and extent of structurally oriented fibrous elements.

RESULTS

Morphology.—The skull of the lamprey consists of a series of incomplete cartilaginous boxes surrounding the brain and organs of special senses, like the auditory, orbital, and nasal capsules. The facial skeleton consists of four main cartilages, the posterodorsal, anterodorsal, posterolateral, and

annular cartilage. The annular cartilage forms the roof of the mouth, where the palatal teeth are attached, and may be considered the jaw of the lamprey. The lip of the lamprey is circular and has a series of papillae which, besides being sensory, serve also to make a tight attachment when the lamprey is attaching itself to its prey. The tongue is attached to a hyoid cartilage through a very long lingual tendon, which continues from the hyoid into a cardioapical muscle attached posteriorly to the cartilage surrounding the heart. Dorsal and ventral to the main tendon are groups of muscles that rock the tongue up and down to produce a rasping action.

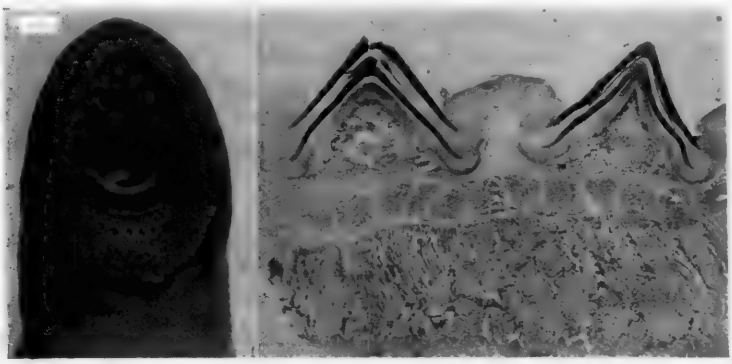

Fig. 1. Fig. 2.

Fig. 1.—The lamprey mouth.

Fig. 2.—Section of buccal cavity and horny "teeth" of the lamprey showing, from top to bottom, the relationship of the primary, secondary, and tertiary horny caps; the stratified squamous epithelium of the oral mucosa; and the underlying connective tissue and muscle layers (unstained ×20; reduced ⅙).

The "teeth" of the lamprey are horny, epidermal thickenings, placed on about twenty tooth-bearing concentric strips (Fig. 1). Every strip has a varying number of teeth decreasing in size toward the periphery. In addition there are about eight sharp, large teeth placed on the tongue. According to their means of fixation they can be divided into teeth with basal cartilaginous nuclei as inner support (lingual and palatinal teeth) and teeth along the mucous membrane which are without any cartilaginous support (labial and buccal teeth). The unsupported teeth are smaller, consisting of a protruded cone of cornified epithelium with small ingrowing connective-tissue papillae. Both show essentially the same structure from outside toward the inside (Fig. 2).

The yellow horn cap is composed of several epithelial layers. It ends sharply in a horny groove submerged in the mucosa. The outer, or primary horny cap is the most intensely cornified part of the lamprey tooth. Below this is a layer of loose, stellate reticulum. It is formed gradually from prickle cells. The lowest cells of this layer are flattened and lie adjacent to the secondary horn cap, forming a cuticle of the secondary cap after its eruption. By continuous use of the tooth, the reticulum degenerates, and

eventually the primary horn cap is worn off, whereupon the secondary horn cap emerges into the mouth. The cone-shaped connective-tissue papilla forms the base of the tooth.

Sulfhydryl Groups and Keratin.—With the Masson triacid stain, it is noted (Fig. 3) that the horny teeth are separated from the underlying muscle by papilla-like projections of connective tissue showing very heavy bundles of green-staining collagen fibers. These collagen fibers become much denser as they infiltrate between the muscle fibers to form the main support of the horny projections. Between this connective-tissue papilla and the horny caps is a layer of epithelial cells which stain much like ordinary epithelium. However, the reticular cell layer, between the primary and secondary caps, is relatively pale except for a thin zone adjacent to the horny caps. From this layer, a reddish staining reaction can be seen at regular intervals through the horny caps as thin lines or bands (Fig. 3). Otherwise the hornified part takes on a yellow homogeneous color. The most intense reddish purple dye is taken up in the epithelial cells underlying the youngest cornified portion, the tertiary cap. The region between this cell layer and the cornified material has a scalloped outline and shows a sudden transition into the more homogeneous yellow-staining horny cap.

With the Ebbinghaus method (Fig. 4), the staining reaction is entirely limited to the hornified caps, primary, secondary, and tertiary alike, all staining yellow—the most mature portion most markedly stained, the young horny groove somewhat paler, especially in proximity to the keratinizing cells.

The method of Chevremont and Frederic (Fig. 5) gives an intense blue reaction in the epithelial layer underlying the youngest horny cap. The caps have a particularly deeply stained layer in the horny grooves of the tertiary cap where the precursors of keratin (SH groups) are being formed, whereas the reaction diminishes toward the more mature parts of the horny secondary and primary caps. In the lamprey, the oral mucous membrane is otherwise relatively unstained with this method, since there is no transformation into keratin.

The sharp distinction in staining reaction of the horny caps noted with the Prussian blue method (Fig. 5) is also reflected in the behavior of this tissue when examined under polarized light (Fig. 6). Here it is noted that the more highly cornified primary and secondary caps are strongly birefringent, whereas the Prussian blue-positive tertiary cap is nonoriented except for a small region at its summit. Presumably this transition coincides with conversion of cysteine to cystine.

This interpretation is further supported by the methods of Barnett and Seligman (Figs. 7 and 8). When both SH groups and disulfide bonds are stained, an intense reaction is evident in all three of the horny caps, as seen in Fig. 7. However, when stained for SH groups alone (Fig. 8), only the tertiary cap is stained. At higher magnification (Figs. 9 and 10), it is noted that the first evidence of cornification is indicated by the appearance of SH-

Fig. 3.—Section stained with Masson's triacid stain shows most marked staining (purple red) of the germinative layer of the horny groove. The primary, secondary, and tertiary horny caps are generally paler (yellowish) with darker (reddish) horizontal bands. Adjacent mucous membrane is not cornified. Underlying tissue shows intensely stained (green) collagen fibers (×50; reduced ⅙).

Fig. 4.—The horny caps are intensely stained (yellow) with the orange II keratin reaction of Ebbinghaus, particularly the older and most highly cornified portions of the caps. Counterstained with hematoxylin (×40; reduced ⅙).

Fig. 5.—The youngest (tertiary) horny cap is intensely stained (blue) with Chevremont and Frederic's Prussian blue reaction for sulfhydryl groups (×50; reduced ⅙).

Fig. 6.—Same section as in Fig. 5 under polarized light showing that SH-positive cap is not structurally oriented except for its summit, a transitional zone, whereas more mature primary and secondary horny caps are highly birefringent (×25; reduced ⅙).

Fig. 7.—Section stained for both SH groups and disulfide bonds with method of Barnett and Seligman shows intense staining of all portions of horny caps regardless of structural orientation (×50; reduced ⅙).

Fig. 8.—Section stained specifically for SH groups according to Barnett and Seligman. Tertiary horny cap shows distinct line of transition between young SH-positive portion and older disulfide-positive birefringent portion. Compare with Figs. 6 and 7 (×50; reduced ⅙).

Fig. 9.—First evidence of cornification is indicated by SH-positive cytoplasmic granules in squamous epithelial cells in deepest layer of the horny groove. Barnett and Seligman's staining reaction (×250; reduced ⅙).

Fig. 10.—As cornification progresses, cellular as well as intercellular regions become SH positive (×250; reduced ⅙).

positive granules in the cytoplasm of the squamous epithelial cells in what is to become the deepest portion of the horny groove. This is followed by a more generalized SH-positive reaction, obscuring and replacing all cellular detail.

Polysaccharides.—When stained with toluidine blue (Figs. 11, 12, and 13), the horny caps remain unstained. A faint metachromatic reaction is present in the connective-tissue ground substance, especially in the basement membrane near the horny groove and in the reticular layer between the horny caps. In the cartilage-supported horny "teeth" of the tongue (Fig. 12), the cartilage shows the expected strong metachromasia of its ground substance. Adjacent to the horny cap there is also an intense metachromatic reaction at the surface of the "gingival" mucous membrane. This is due to an accumulation of the mucous substance in the cytoplasm of large mucous cells (Fig. 13). At a distance from the horny teeth, the mucous membrane is relatively unstained by this method except for the basophilic staining of the nuclei.

The basophilic nature of the metachromatic staining reaction was evident from staining similar sections with methylene blue at various levels of pH (Fig. 14). With this means, an intensely basophilic reaction could be observed in the mucous epithelial cells at a pH of 3, even though at this point the nuclear basophilia of the basal epithelial cells have much diminished or disappeared. In other words, the nucleoproteins are no longer stained, whereas the metachromatic material is of sufficiently acidic character to have stainability at this low pH.

The basophilia of the ground substance of the connective-tissue papilla and that of the stellate reticulum are much diminished or abolished at low pH, whereas the ground substance of the underlying cartilage core in the horny teeth of the tongue retains an intense metachromasia as well as basophilia at this pH. This combination of metachromatic and basophilic staining properties is designed to locate the presence of acid mucopolysaccharide.

With the periodic acid-Schiff method (Fig. 15), there is an intense reaction in the stratum granulosum of the epithelial layers ascribed to the presence of glycogen. This reaction is, however, not particularly conspicuous in the epithelium bordering on the developing horny caps. The reaction is faint in the stellate reticular layer but is quite noticeable in the ground substance of the underlying connective-tissue papilla.

Alkaline phosphatase appears to be most abundant in the reticular cell layer between the horny caps, especially in the layer adjacent to the primary horny caps (Fig. 16). Here the reaction is evident at first in the nuclei (Fig. 17) and then increasing in the intercellular substance. In the underlying secondary and tertiary horny caps themselves, the reaction is not present. In the epithelium underlying the deeper horny caps, the reaction is relatively faint. However, in the cells surrounding the horny grooves (Fig. 18), the reaction is particularly intense in the cell nuclei, with a faint stippling in the intercellular substance and a pale inactive zone in the cytoplasm bordering on the nuclei.

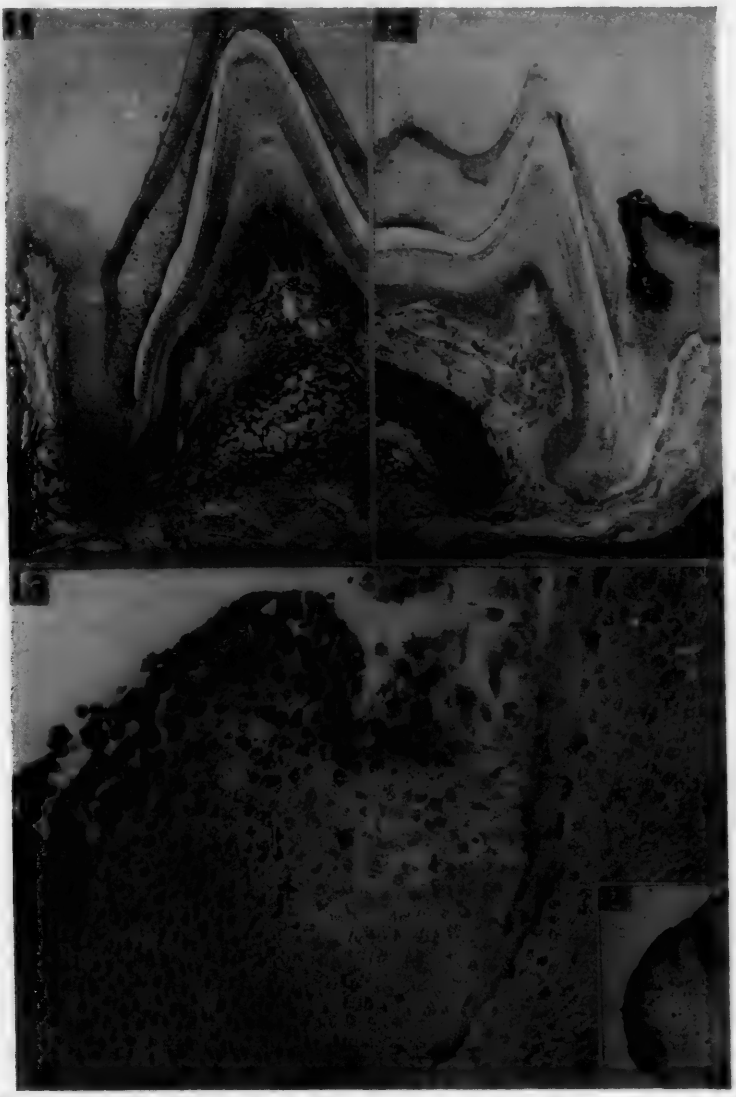

Fig. 11.—Toluidine blue-treated section reveals metachromatic reaction of ground substance of connective tissue, particularly in region of basement membrane. Germinative layer adjacent to horny groove and reticular layer between horny caps also show faint metachromatic reaction (×40; reduced ⅙).

Fig. 12.—Toluidine blue-stained section of cartilage-supported horny caps of tongue. Most intense metachromatic reaction is visible in the central segment of cartilage and in mucous surface cells adjacent to horny cap (×40; reduced ⅙).

Fig. 13.—Higher magnification of Fig. 12 showing intensely metachromatic mucus formed by epithelial cells in near proximity to unstained cornifying epithelium of horny cap (×250; reduced ⅙).

Fig. 14.—Section from same region as in Fig. 13 stained with methylene blue at pH 3.0. At this low pH, the nuclear reaction is faint, while there is strong basophilic reaction at site corresponding to metachromatic mucus (×40; reduced ⅙).

In the cells of the underlying connective tissue papilla, the alkaline phosphatase reaction is quite marked in the nuclei and around blood vessels but generally not as intense as in the stellate reticular layer underlying the primary horny cap. The epithelium covering the mucous membrane is quite pale when stained by this method. The control sections failed to stain, indicating that there is little or no calcium phosphate deposited in the horny teeth of the lamprey.

Lipid.—Frozen sections stained with sudan IV show extensive deposits intermuscularly and also in the basal portion of the connective-tissue papilla which projects into the core of the horny caps. In addition, there is a lipid reaction in the reticular cell layer and a stippling toward the functional ends of the horny caps, where it eventually becomes completely negative (Fig. 19). Aside from the tissue surrounding the horny cap and the intercellular bridges, the mucous membrane appears unstained by this method.

DISCUSSION

We have noted that the squamous epithelial cells of the oral mucosa of the lamprey are capable of producing a mucous product in near proximity to those which produce the keratinous framework of the horny caps. In higher animals the mucous secretion is formed by the numerous mucous glands submerged in the mucosa of the lips, cheeks, and palate, whereas the enamel-forming cells, the ameloblasts, are endowed with a dual capacity within one and the same cell: first, to produce a mucopolysaccharide ground substance into the stellate reticulum of the enamel organ, and then, following reversal of the polarity of the cells, to secrete a keratinous component into the primordium of the enamel prisms.*

The epithelial and mesenchymal cells which give rise to the horny caps and their connective-tissue papillae fail to differentiate into cells comparable to the highly specialized odontogenic cells. Their products, the keratinous and collagenous fibers, respectively, remain uncalcified. In higher animals, the enamel not only gives a keratinous reaction, but a mucopolysaccharide appears to enter the enamel matrix just before calcification becomes a prominent feature.[16] Furthermore, it is well known that the reaction for alkaline phosphatase is extremely intense in the odontogenic tissues, especially in the ameloblastic and odontoblastic cell layers. This is in keeping with the theory that an important function of this enzyme is to split off organically bound phosphate to make it available for the calcium deposited in the enamel and dentin during calcification. On the other hand, it is also evident that the alkaline phosphatase reaction is similarly conspicuous in the epithelial cells adjacent to the horny groove of the lamprey teeth, even though the process of calcification can here be completely ruled out. Whereas epithelial coverings of lower animals are quite capable of taking up calcium carbonate,[3] calcium phosphate begins to become a more conspicuous component in the claws of some crustaceans[4] and is the dominant mineral component of the

*A similar chemical sequence has been observed in the vaginal epithelium, which alternately produces a mucous and keratinous substance during the estrus cycle.[16]

Fig. 15.—Schiff's periodic acid reaction is most marked in stratum spinosum and cells adjacent to horny groove presumably due to presence of glycogen (×40; reduced ⅛).

Fig. 16.—Alkaline phosphatase reaction is extremely intense in stellate cells between horny caps as well as in squamous cells adjacent to horny grooves (×40; reduced ⅛).

Fig. 17.—Higher magnification of middle segment from Fig. 16 shows alkaline phosphatase adjacent to primary (left) and secondary (right) caps (×250; reduced ⅛).

Fig. 18.—Horny groove enlarged from Fig. 16 showing intense nuclear reaction for alkaline phosphatase adjacent to the site of the cytoplasmic SH reaction (compare with Fig. 9). More widespread cellular and intercellular phosphatase reaction is noted adjacent to more advanced cornification (compare with Fig. 10). (×250; reduced ⅛.)

Fig. 19.—Frozen section stained with sudan IV. Sudanophilic reaction is distinct in degenerating pyknotic cells of cornifying epithelium and faintly visible in the reticular cells adjacent to horny caps (×250; reduced ⅛).

enamel in higher animals. The absence of calcification of the horny caps of the lamprey can obviously not be attributed to the absence of alkaline phosphatase reaction in the adjacent cells. Similarly we have noted the presence of a mucopolysaccharide ground substance, alkaline phosphatase, and a collagen framework in the underlying connective-tissue papilla of the lamprey teeth, altogether without calcification.

These various combinations, in which calcium phosphate is absent despite the presence of compounds usually associated with calcification, point to the importance of additional factors or substances, possibly in the form of other enzymes, which have a more specific function in bringing about the deposition of calcium phosphate. The histochemical reactions accompanying the initial appearance of these specialized crystals in the course of evolution would seem worthy of further exploration.

SUMMARY

1. A study has been made of the morphology of the lamprey mouth and the histochemistry of the keratinous and mucous products of the squamous epithelial lining of the oral mucosa.

2. An increasing degree of cornification has been demonstrated from the young tertiary caps to the functional primary caps of the lamprey teeth.

3. The beginning of this transformation is evidenced by the appearance of SH-positive granules in the cytoplasm of the squamous epithelial cells.

4. The appearance of these granules is accompanied by an intense alkaline phosphatase reaction in the nuclei of the squamous epithelial cells.

5. As cornification progresses from the tertiary to the secondary and primary caps, transition from SH groups to disulfide bonds can be demonstrated.

6. The transition from SH to disulfide reaction is accompanied by an increasing structural orientation of the fibrous protein. This is evidenced by a brilliant birefringence when the secondary and primary caps are examined under polarized light.

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GLYCOGEN CONTENT OF PERIODONTAL TISSUE

II. Correlation With Tyrosine Content

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IN A recently reported study[1] on the glycogen content of gingival tissue in chronic periodontitis, it was found that tissue adjacent to shallow pockets had a higher glycogen content than normal gingival tissue and that this glycogen level decreased with increasing pocket depth. While this chemical finding could be correlated with histologic studies in periodontitis, some uncertainty remained because of the method used to determine sample weight. This paper reports the results of an additional study designed to consider this question.

For reasons described earlier,[1] the weights of the original samples of gingival tissue were determined by a chemical wet-ash method.[2] This spectrophotometric procedure is based on the reduction of acid dichromate by the oxidizable organic material in the sample. If the proportion of this organic material to the total mass of the sample remains constant, the actual weight can be obtained from a suitable calibration curve. However, if the composition of the tissue changed, the calibration curve would be worthless. In the experimental work on gingival tissue from regions of periodontal pathology there was a possibility of influx of edema fluid which would give false estimations of sample weight erring on the low side and thus lead to false glycogen percentages on the high side. It was felt desirable to investigate this possibility by introducing into the routine a third analytic procedure to estimate a second tissue component not likely to be altered by the pathologic condition and which could therefore be used as a check on the weight of the sample. Edema fluid contains only small quantities of protein, and so it was decided to analyze for tyrosine, a common protein constituent, for which there is a relatively simple and specific method. Thus an estimation of protein levels in the samples would be obtained. If the tyrosine content of the tissues remained constant, then there would be no question of inaccuracy in sample weights.

EXPERIMENTAL

Patients exhibiting periodontal pockets of varying depths or isolated pockets with apparently normal tissue adjacent to the pathologic region were selected. The desired sites were anesthetized with Xylocaine hydrochloride,

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and the papilli adjacent to the pockets were excised, giving samples of approximately 20 to 30 mg. Each sample was briefly rinsed in water to remove saliva and excess blood and then was placed in 1 ml. of 30 per cent KOH and heated in a boiling water bath until solution of the sample was complete. An additional 1 ml. of water was added to each solution, and then aliquots of 0.2 ml., 1.0 ml., and 0.5 ml. were taken for estimations of weight (wet ash),[2] glycogen,[3] and tyrosine,[4] respectively.

The first two procedures were carried out as reported earlier.[1] For the tyrosine determination, the 0.5 ml. aliquot was diluted with 10 ml. of water, and 0.5 ml. of Folin's phenol reagent[4] was added. The solution was already sufficiently alkaline because of the KOH content of the sample solution. After standing for thirty minutes at room temperature for color development, the samples were centrifuged and the supernatant fluid read in a Klett-Summerson photometer at 540 mμ. Tyrosine content was obtained from a calibration curve prepared from standard tyrosine solutions.

In order to establish the precision of the individual analytic methods, a large sample of gingival tissue (185 mg.) was obtained, weighed on an analytic balance, dissolved in 12.5 ml. of 30 per cent KOH, and diluted to 25 ml. in a volumetric flask. Aliquots of this stock solution were analyzed in replicate by each method.

TABLE I
SUMMARY OF ANALYTIC RESULTS

SUBJECT	SAMPLE	POCKET DEPTH (MM.)	% GLYCOGEN	% TYROSINE	MG. GLYCOGEN / MG. TYROSINE
A	1	3	0.552	1.35	0.410
	2	3	0.253	1.24	0.204
	3	3	0.335	1.17	0.287
	4	1.5	0.227	1.24	0.184
	5	3.5	0.211	1.26	0.167
	6	3	0.221	1.23	0.180
	7	3	0.158	0.81	0.195
	8	1	0.095	1.10	0.086
B	9	1	0.185	1.13	0.165
	10	7	0.138	1.13	0.122
	11	7	0.061	1.09	0.055
	12	7	0.122	1.06	0.116
C	13	2	0.270	1.03	0.183
	14	5	0.271	1.19	0.228
	15	3	0.273	1.01	0.270
D	16	2	0.145	0.51	0.283
	17	3	0.206	0.90	0.233
	18	3	0.171	0.95	0.180
E	19	3	0.125	1.05	0.119
	20	5	0.128	1.04	0.123
	21	5	0.112	0.94	0.120
	22	5	0.193	1.19	0.163
F	23	5	0.227	0.99	0.229
	24	4	0.133	0.93	0.144
	25	7	0.159	1.18	0.135
			Mean	1.07	
			Standard deviation	±0.17	

RESULTS

The pertinent analytic results, twenty-five samples representing six patients, are given in Table I. It can be seen that, while glycogen levels vary widely from sample to sample, tyrosine levels remain markedly constant. No mean value for glycogen is given since it has already been shown[1] that this is a variable factor. Since the tyrosine percentages remain constant, it should be possible to deduce the original sample weight from the result of the tyrosine analysis. A more direct approach would be to deduce the glycogen content directly from the chemically estimated values for total tyrosine and total glycogen. From the data, it is possible to calculate the desired equation since a straight-line relationship exists between the ratio mg. glycogen/mg. tyrosine and per cent glycogen with a correlation coefficient, $r = 0.877$. This equation is calculated to be:

$$\text{Per cent glycogen} = -0.0084 + 1.1338\ G/T$$

where G = total mg. glycogen, T = total mg. tyrosine.

From the standard deviation of the intercept, it can be shown by statistical analysis that the intercept is not significantly different from the origin ($t = 0.34$ for 24 d.f.). Therefore the relationship becomes:

$$\text{Per cent glycogen} = 1.1338\ G/T.$$

In the replicate series, eight tyrosine and eight glycogen determinations were performed. The tyrosine series had a mean of 1.16 per cent with a standard deviation of ±0.13 and the glycogen, a mean of 0.132 per cent with a standard deviation of ±0.023, showing both methods to be satisfactorily precise.

DISCUSSION

Since gingival tissue tyrosine remained constant while the glycogen varied, it is apparent that the observed glycogen variation was real and not an artefact resulting from the determination of sample weight by the wet-ash method. Had the variations in glycogen reported previously,[1] and confirmed here, been the result of false values for sample weight due to edema fluid in the tissue, the tyrosine content should have been found to vary in a manner similar to the glycogen.

In the light of the constant tissue tyrosine content, and considering the precision of the tyrosine analysis as compared to the indirect and uncertain wet-ash technic, it now appears more desirable, when working with small samples of gingival tissue, to use the analytic value for tyrosine as a measure of the size of the original sample. The equation presented previously allows calculation of per cent glycogen when total milligrams glycogen and total milligrams tyrosine are known.

SUMMARY

1. In order to determine whether the variations found in glycogen levels in gingival tissue in various stages of periodontal disease were real or due to experimental artefact, gingival tissue was analyzed for glycogen and tyrosine.

2. Tyrosine levels were found to be constant, while glycogen levels varied, indicating that the glycogen variations are true findings.

3. An equation was derived relating per cent glycogen to total milligrams glycogen and total milligrams tyrosine in the sample.

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